University of Alberta

The Role of Neuroinflammation in Cell Death and Cell Survival after Neonatal Hypoxia-Ischemia

by

Lauren Leigh Cooney Jantzie ©

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ABSTRACT

Many developmental, neurological and psychiatric disorders (i.e. cerebral palsy, mental retardation, and learning disabilities) have been linked to birth trauma, disruption in the maternal-placental-fetal system and hypoxia-ischemia (HI) in early life. HI leads to significant neuroinflammation as evidenced by increased microglial activation, proinflammatory cytokine release, increased matrix metalloproteinase activity and profuse neuronal cell loss. The tetracycline derivative doxycycline (DOXY) has anti-inflammatory actions independent of its antimicrobial effects and has been reported to be neuroprotective in adult animal models of cerebral ischemia. To investigate the role of neuroinflammation in cell death and cell survival after neonatal HI and the consequences of DOXY administration, a timecourse study was run such that neonatal rats received DOXY (10 mg/kg) or vehicle (VEH) ($n \geq 6$) in clinically relevant dosing regimes. Pups were euthanized 30 minutes to 7d post-HI. Immunochemical methods were used to detect specific cell markers, inflammatory cytokines, markers of cell genesis, and cell death signaling proteins. High performance liquid chromatography was used to determine levels of DOXY in brain parenchyma and concentrations of amino acid neurotransmitters. In addition to showing that DOXY significantly penetrates the blood- brain barrier, the studies presented here demonstrate that neuronal cell loss and evidence of neuroinflammation was present throughout the brain of all HI pups. Treatment with DOXY significantly attenuated this cell loss, robustly inhibited microglial activation, decreased the levels of interleukin-1β and tumor necrosis factor alpha, augmented the levels of brain derived neurotrophic factor, and decreased cleaved caspase-3 protein expression (all

$p<0.05$). HI also significantly altered brain levels of the amino acids investigated including glutamate, γ-aminobutyric acid, alanine, serine, and glycine. Treatment with DOXY significantly normalized levels of alanine, serine, and glycine in every region examined, 4h post-HI ($p<0.05$). Compared to VEH-treated pups, DOXY-treated pups had fewer 2-bromo-5-deoxyuridine positive cells in the subventricular zone immediately following HI, but DOXY administration did not persistently affect the timecourse of neurogenesis in the subventricular zone or dentate gyrus 7d post-HI. This thesis highlights the complex role of activated microglia in cell survival and cell death following HI and a putative role for DOXY in the treatment of the brain damage associated with HI.

DEDICATION

To my Grandparents, Chris and Edna Cooney, who along with my parents, were my first teachers, my first role-models and my first fans. It is because of the timeless gifts they have given me that I dedicate this thesis to them. Two people with fabulous minds, incredible spirits and the most beautiful hearts I have ever known.

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LIST OF ABBREVIATIONS

ADHD	attention deficit hyperactivity disorder
ADP	adenosine diphosphate
AIF	apoptosis-inducing factor
ALA	alanine
ANOVA	analysis of variance
AMP	adenosine monophosphate
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole
Apaf-1	apoptotic protease-activating factor adaptor protein
ASN	asparagine
ASP	aspartate
ATP	adenosine triphosphate
BBB	blood-brain barrier
Bcl	B-cell lymphoma
b.i.d.	*bis in die*, twice daily
BCA	bicinchoninic acid
BDNF	brain-derived neurotrophic factor
BrdU	5-bromo-2-deoxyuridine
BSA	bovine serum albumin
Ca^{2+}	calcium
CA1	*cornu ammonis* 1 (hippocampal subfield)
CA3	*cornu ammonis* 3 (hippocampal subfield)
CBF	cerebral blood flow

cc	cubic centimetre
CCA	common carotid artery
CC3	cleaved caspase-3
CK-BB	serum creatine kinase brain bound
CNPase	2', 3'-cyclic nucleotide 3'-phosphodiesterase
CNS	central nervous system
CNTF	ciliary neurotrophic factor
COX-2	cyclooxygenase-2
CSF	cerebral spinal fluid
CT	computerized tomography
Cy2	cyanine
d	day
DAB	3,3'-diaminobenzidine
DG	dentate gyrus
DNA	deoxyribonucleic acid
DOXY	doxycycline
DR4	death receptor-4
DR5	death receptor-5
ECAM-1	endothelial cell adhesion molecule-1
ECL	electrochemiluminescence
ECM	extracellular matrix
ECMO	extracorporeal membrane oxygenation
EEG	electroencephalogram

ELISA	enzyme-linked immunsorbent assay
EPO	erythropoietin
FITC	fluorescein isothiocyanate
g	gram
GABA	γ-aminobutyric acid
GABA-T	γ-aminobutyric acid transaminase
GAD	glutamic acid decarboxylase
GDNF	glial-derived neuroptrophic factor
GFAP	glial fibrillary acidic protein
GLN	glutamine
GLU	glutamate
GluR	glutamate receptor
GLY	glycine
GLYT-1	glycine transporter-1
GZ	gelatin zymography
h	hour
HI	hypoxia-ischemia
HIE	hypoxic-ischemic encephalopathy
HPLC	high performance liquid chromatography
HRP	horseradish peroxidase
HUS	head ultrasonography
ICAM-1	intracellular adhesion molecule-1
ICE	interleukin-1β converting enzyme

ICH	intracranial hemorrhage
IFNγ	interferon gamma
IgG	immunoglobulin G
IL-1β	interleukin-1β
IL-6	interleukin-6
iNOS	inducible nitric oxide synthase
IUGR	intrauterine growth retardation
IUI	intrauterine infection
K^+	potassium
kDa	kilo dalton
Ki-67	antigen identified by monoclonal antibody Ki-67 or MKI67
M	molar
mM	millimolar
MAPK	mitogen activated protein kinase
MCAO	middle cerebral artery occlusion\
min	minute
MINO	minocycline
mL	millilitre
MMP	matrix metalloproteinase
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
MRSA	methecillin resistant *Staphylococcus aureus*
N	normal

NAD^+	nicotinamide adenine dinucleotide
NeuN	neuronal nuclei/neuronal specific nuclear protein
ng	nanogram
NGF	nerve growth factor
nM	nanomolar
NO	nitric oxide
NOS	nitric oxide synthase
NICU	neonatal intensive care unit
NMDA	N-methyl-D-aspartate
NT-3	neurotrophin-3
NT-4/5	neurotrophin-4/5
OPA	σ-phthaldialdehyde
P	post-natal day
PAIS	perinatal arterial ischemic stroke
PARP	poly(ADP) ribose polymerase
PBS	phosphate-buffered saline
PCr	phosphocreatine
PECAM	platelet endothelial cell adhesion molecule-1/CD-31
PMN	polymorphonuclear cells
PSA-NCAM	polysialylated embryonic neural cell adhesion molecule
PVDF	polyvinylidene fluoride
PVL	periventricular leukomalacia
rcf	relative centrifugal force

RNA	ribonucleic acid
SD	SHAM + DOXY
sd	standard deviation
SDS-PAGE	soldium dodecyl sulfate polyacrylamide gel electrophoresis
SER	serine
SVZ	subventricular zone
TAUR	taurine
TEA	triethylamine
TGFβ1	tumor growth factor beta-1
THF	tetrahydrofuran
t.i.d.	*ter in die*, thrice daily
TMB	tetramethylbenzidine
TNFα	tumor necrosis factor alpha
tRNA	transfer ribonucleic acid
UV	ultraviolet
V	volts
v	volume
VCAM-1	vascular cell adhesion molecule-1
VEGF	vascular endothelial growth factor
VEH	vehicle (saline)
VRE	vancomycin resistant enterococcus
w	weight
μg	microgram

μl	microlitre
μM	micromolar

CHAPTER 1

Neonatal Hypoxia-Ischemia & Doxycycline: Basic Science and Clinical Perspectives

1.1. Neonatal Hypoxia-Ischemia

Neonatal or perinatal hypoxia-ischemia (HI) is a common clinical occurrence accounting for at least 16% of deaths in Canadian neonatal intensive care units (NICUs) (Sankaran *et al.* 2002). HI is a specific, descriptive term used to explain conditions of reduced partial pressure of oxygen in the blood and complete cessation of blood flow to a certain area of the body, organ or tissue. Fetal (before birth), perinatal (20 weeks gestation up to post-natal-day 7), and neonatal (birth up to 28 days post-delivery) HI has a wide range of presentations and manifestations, and affects the heart, lungs, gastrointestinal tract, kidneys and brain most severely. Infants that survive HI often exhibit symptoms that reflect a complex multi-system disorder, with multi-organ failure. In addition to significant changes in hemodynamics, acute renal failure, respiratory failure, pulmonary hemorrhage, pulmonary edema, meconium aspiration syndrome, papillary muscle necrosis, cardiovascular shock, gastrointestinal hemorrhage, necrotizing enterocolitis, hyperammonemia, and hypoxic-ischemic encephalopathy (HIE) are commonly diagnosed post-HI (Sunshine 2003, Carter *et al.* 1993).

Multiorgan dysfunction is a distinguishing feature of severe neonatal HI situations and this phenomenon is mechanistically related to the diving reflex (Shah *et al.* 2004). The diving reflex shunts blood away from less vital organs such as the skin, to redistribute and redirect blood flow to protect the heart, adrenal glands and brain. Thus, it is very likely that every neonate with clinically detectable heart and/or brain dysfunction following HI would have activated the diving reflex for long enough to damage other organs, particularly the kidneys, gastrointestinal tract and liver (Shah *et al.* 2004, Roland & Hill 1997). This is why almost every infant that survives an HI insult has multiple comorbid medical conditions. However, the neurological complications sustained post-HI are amongst the most devastating. Infants that survive HI injuries often manifest neurodevelopmental and neuropsychiatric disorders including, but not limited to: disorders of neuronal migration, learning disabilities, conduct disorders, mental retardation, seizure disorders, cerebral palsy, hearing and vision loss, and attention deficit hyperactivity disorder (ADHD). It is because these neurologic complications are severe, frequent, pervasive, and debilitating, that the focus here is on the brain damage associated with HI events. Although the focus of this thesis is neonatal cerebral HI, it is duly recognized that the neurologic complications resulting from HI represent only one facet of an exceedingly complex, multi-system disorder with many devastating consequences.

1.1.1. Defining Neonatal Hypoxia-Ischemia

Hypoxic-ischemic brain injury is specifically defined as neuropathology attributable to hypoxia and/or ischemia as evidenced by biochemical (serum creatine kinase brain bound [CK-BB]), electrophysiologic (EEG), neuroimaging (head ultrasonography [HUS]), magnetic resonance imaging (MRI), computed tomography (CT) or postmortem abnormalities (Adcock & Papile 2008). Although there has been a marked reduction in morbidity and mortality rates over the past four decades, HI in the perinatal period leading to major motor and cognitive disabilities continues to be a significant health problem world wide (Sunshine 2003). Further, the mortality from acute neurologic disorders of childhood is highest in infants under one year of age (Maytal *et al.* 1989, Fullerton *et al.* 2002, Ferriero 2004). Despite advances in fetal monitoring including biophysical profiling, 2D & 3D ultrasound, and *in utero* MRI, a large number of infants with neurological abnormalities are born each year, many due to problems encountered during pregnancy and the birthing process (Sunshine 2003). Although the neonatal brain constitutes only 5-10% of the body's mass, it uses 20% of the body's oxygen supply and receives 15% of total cardiac output. As such, it is extraordinarily vulnerable to changes in blood flow and oxygen concentration. Often, colloquialisms such as stroke or asphyxia are used interchangeably to describe cerebral HI, but their clinical definitions are diverse. Asphyxia refers to the complete cessation of placental or pulmonary gas exchange with progressive hypoxemia, hypercapnia and metabolic acidosis. Stroke is defined as "a sudden and severe attack" (Dorland's 2001) and refers to a syndrome reflecting the infarction of vascular territory put at risk by an interruption in blood flow. As this is somewhat ambiguous and because the terminology used to describe the depressed or affected infant are often vague, HI will be the terminology used throughout this thesis as it is more descriptive and offers greater specificity in describing specific events occurring in the perinatal/neonatal period.

1.1.2. Ischemic and Hemorrhagic Stroke in Infants

Ischemic and hemorrhagic strokes are two manifestations of HI brain injury known to affect the pediatric population. Many strokes in children happen in the perinatal period, soon before birth or within the month after (Nelson & Lynch 2004). Pure ischemic strokes (those that occur as a result of a thrombus or embolus) occur most often in older children or chronically instrumented neonates with congenital heart abnormalities or severe coagulopathies (Perlman 2004). As is typically the case in older children, there is no symptom prodrome or acute illness associated with these events, and they are most often related to previously undiagnosed medical conditions or trauma. Hemiparesis is primarily the chief compliant upon presentation in the emergency room. In newborns, the most common occlusive events are sinovenous thrombosis and perinatal ischemic arterial stroke (PAIS) (Andrew *et al.* 2001). PAIS is among the most common causes of hemiplegic cerebral palsy (Golomb *et al.* 2007). Spontaneous focal infarcts in the neonatal period are rarely encountered, but may be associated with maternal drug abuse, septicemia with or without meningitis, maternal diabetes, and polycythemia (Sunshine 2003, Jan & Camfield 1998, Gunther *et al.* 2000). The heightened vulnerability to stroke and to thromboses in non-cerebral sites is most likely related to activation of coagulant mechanisms by parturition, presumably an evolutionary adaptation to lessen the risk of hemorrhage at this crucial time (Suarez *et al.* 1985, Nelson & Lynch 2004, Schmidt & Zipursky 1984, Lao *et al.* 1990). Intracranial hemorrhage (ICH) in the full-term infant is less common than in premature infants, but infants of any age can manifest hemorrhages of the subdural, subarachnoid, intraventricular and perenchymal variety (Shankaran 2003, Perlman 2004). In the immature brain, hemorrhage can also occur as a result of specific conditions such as arteriovenous malformations, neonatal alloimmune thrombocytopenia, periventricular leukomalacia (PVL) and/or after exposure to extracorporeal membrane oxygenation (ECMO) (Shankaran 2003). Hemorrhagic strokes can also occur after trauma, although the incidence of ICH attributed to birth trauma has been declining since perinatal care has improved and there is increased recognition that not all lesions of this type are related to the delivery process (Perlman 2004). However, with increased use and wider availability of prenatal ultrasonography, the occurrence of *in utero* subdural hemorrhage has been documented with greater frequency (Gunn *et al.* 1985, Perlman 2004, Towbin 1970).

1.1.3. Etiology of Hypoxic-Ischemic Brain Damage

In term infants, 90% of severe HI events occur in the antepartum or intrapartum period as a result of impaired gas exchange across the placenta, leading to inadequate provision of oxygen, and removal of carbon dioxide and H^+ from the fetus (Adcock & Papile 2008). In general, HI is most easily understood as acute or chronic disruption in the maternal-placental-fetal system. Sentinel events related to pregnancy that acutely cause fetal brain injury include: placenta previa, micro-placentation, abruptio placentae, placental infarction, placental hydrops, umbilical knotting, umbilical prolapse, umbilical entanglement, umbilical compression, uterine rupture, amniotic fluid embolism, acute neonatal hemorrhage (vasa previa, acute blood loss from cord), acute maternal hemorrhage, severe anemia and/or any condition causing abrupt decreases in maternal cardiac output and or blood flow to the fetus (Adcock & Papile 2008, Sunshine 2003). Specific conditions of pregnancy can also render a fetus more vulnerable to HI. For example, there is a greater risk for HI in multiple gestation pregnancies than singleton pregnancies, and twin-twin transfusion syndrome is paramount in these settings. Intrauterine growth retardation (IUGR), or chronic HI, is often the major consequence of twin-twin transfusion syndrome. Chorioamnioitis, intrauterine infection, certain coagulopathies and metabolic disorders are also associated with an increased risk of HI. Direct trauma to the fetal head and neck during labor and delivery, as sometimes observed in vacuum extraction and forceps-assisted deliveries can also be associated with increased HI risk. After delivery, seriously ill neonates may become spontaneously HI as is commonly observed in NICUs. These neonates have multiple medical problems, medications and instrumentation required for life-support. Critically ill infants are more susceptible to fluctuations in systemic blood pressure, have increased propensity to form clots, and have impaired cerebral autoregulation, thus rendering them more vulnerable to HI (McLean & Ferriero 2004).

As highlighted above, certain events occurring during the normal course of labor can decrease oxygen reserve in the neonate. Examples of these events include: 1) decreased blood flow to the placenta and fetal head due to uterine contractions; 2) cord compression; 3) maternal dehydration; 4) maternal alkalosis due to hyperventilation; 5) reduction in placental blood flow; and 6) increased oxygen consumption by both mother and fetus (Adcock & Papile 2008). Despite all of these factors, the immature brain is inherently resistant to HI because of innate adaptations to the intrauterine environment. For example, compensatory increases in fetal cerebral blood flow (CBF) are usually capable of maintaining oxygen delivery to meet demand. However, injury to brain tissue occurs when CBF is decreased beyond threshold.

During a labor complicated by a HI challenge, the severity of the resultant brain lesion depends on many variables, including: the nature and duration of the episode, the gestational age of the fetus, the presence/absence of superimposed systemic stress such as infection, and the amount of collateral blood flow to the affected areas of the brain. Chorioamnionitis, funisitis and intrauterine infection (IUI) may be comorbid in some cases of HI and are commonly associated with delivery room depression and neonatal encephalopathy. Typically in IUIs, microbes such as group B *Streptococcus* and *Escherichia coli* ascend from the lower maternal genital tract to the decidual lining of the amniotic sac and ultimately, the amniotic fluid. As a result, the immature immune system mounts a significant immune response to combat infection and this peripheral and central inflammation in the fetus interacts synergistically with placental cytokine release and intrauterine inflammation. In the presence of infection, HI brain lesions are capable of expanding to volumes and regions beyond what may have occurred in the presence of either infection or HI alone.

1.1.4. Developmental Vulnerability

Many adult diseases have their origins in prenatal or early postnatal life, part of a phenomenon collectively known as fetal/neonatal programming. Further, prenatal factors are important predictors of long-term neurologic outcome. HI injury alters the trajectory of normal developmental events and begins a cascade for altered maturation. As such, delineating the mechanisms which underlie the vulnerability of the developing central nervous system (CNS) to diverse insults is vital to improving both the diagnosis and treatment of a multitude of disorders. Part of the reason why HI is so detrimental is that it is an injury that occurs in developing organs, on a background of natural and necessary cell death. Further, molecules that have important roles in the pathogenesis of HI (i.e. iron, glutamate, calcium, and nitric oxide) are often mediators of crucial developmental possesses (McLean & Ferriero 2004). The final neurological pattern that exists after HI (both radiographically and neurodevelopmentally) depends partly on the gestational age at which the insult occurred (McLean & Ferriero 2004). In the immature CNS, neurons are selectively vulnerable in a manner that is comparable to cell-type vulnerabilities in adult neurological disorders like Parkinson's disease and Huntington's disease (Ferriero 2004). This concept of selective vulnerability of different brain structures and neural cells at different stages of development also determines the immature CNS's response to HI (McLean & Ferriero 2004).

Common neurologic manifestations of HI in premature infants (infants born less than 37 weeks gestation) are severe damage to hemispheric white matter with relative sparing of the grey matter (Squier & Cowan 2004). However, acute and severe damage to the basal ganglia and thalamic nuclei are also occasionally observed (Squier & Cowan 2004). The most common post-HI diagnoses made in premature infants are PVL and periventricular hemorrhagic infarction. PVL is hallmarked by a characteristic pattern of white mater injury defined by infarcts, with or without cysts, in periventricular and callosal white matter and watershed zones of end artery distributions. Increasing experimental and clinical data support the notion that the infant's brain possesses many fundamental differences in function, cellular composition, and connectivity compared to the adult brain (Jensen 2006). As such, it is believed that PVL and white matter injuries are most commonly seen in preterm infants because developing oligodendrocytes and subplate neurons are preferentially vulnerable (Ferriero 2004, McQuillen & Ferriero 2004, McQuillen *et al.* 2003). Damage to subplate neurons is believed to be involved in the vision loss sometimes associated with HI, as these cells play a critical role in the formation of connections between the thalamus and the visual cortex (Kanold *et al.* 2003).

In addition to intrinsic properties of cellular CNS populations, the premature white matter is rendered particularly vulnerable due to the immaturity of penetrator blood vessels into deep cortex and white matter, as well as lack of autoregulatory control (Jensen 2006, Soul *et al.* 2000). Thus, when systemic hypotension occurs, there is little compensatory regulation in these regions (Jensen 2006). The age window of greatest susceptibility to PVL is between 24-32 weeks gestation, when the cerebral white matter is predominantly populated by oligodendrocyte precursors and immature oligodendrocytes (Jensen 2006, Back *et al.* 2001, Kinney & Back 1998). Preoligodendrocytes and oligodendrocyte progenitor cells are more vulnerable to HI than mature oligodendrocytes. This is largely thought to be due to differences in the capability of these cells to cope with cellular factors such as oxidative stress and excitotoxicity. For example, the immature brain has high concentrations of unsaturated fatty acids and redox-active iron, a high rate of oxygen consumption, and low concentrations of antioxidants (Ferriero 2004). As a result, premature infants with many more oligodendrocyte progenitors and preoligodendrocytes, are extremely vulnerable to the depletion of antioxidants or exposure to exogenous free radicals. As infants approach term and the population of mature oligodendrocytes grows, the baby and its brain cells become more resistant to oxidative stress. This is largely thought to occur as a result of the increases in anti-oxidant enzymes that occur with age (Ferriero 2004).

Oligodendrocyte precursors are also extremely susceptible to excitotoxicity and owe their preferential vulnerability to differences in α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor subunit composition (Talos *et al.* 2006a, Talos *et al.* 2006b). *In vivo*, glutamate receptor subunit-2 (GluR2) deficient- AMPA receptors are highly expressed in immature and premyelinating pre-oligodendrocytes (Jensen 2006). AMPA-mediated signaling and excitotoxicity depend on the functional properties of the receptor complex, such as calcium permeability, which in turn are dictated by subunit composition (Deng *et al.* 2003, Follett *et al.* 2004, Talos *et al.* 2006a, Talos *et al.* 2006b). AMPA receptors lacking the GluR2 subunit are calcium permeable, and because these are the most abundant receptor subtype on immature oligodendrocytes, it is believed that they are the reason for the described developmental vulnerability to excitotoxicity and white matter damage.

In term infants (32-42 weeks gestation), a single profound total failure of blood or oxygen supply (i.e. cardiac arrest) typically causes symmetrical damage to the nuclei of the brainstem, including the cranial nerve nuclei and thalami (Squier & Cowan

2004). Many of these infants do not survive the insult and those who do show disorders of thermoregulation, sucking, swallowing and oculomotor paresis (Squier & Cowan 2004, Felderhoff-Mueser *et al.* 1999, Pasternak & Gorey 1998). Slightly less acute and profound HI allows time for fetal adaptive responses and blood is redistributed to the vital centres of the brainstem and shunted away from the hemispheres (Squier & Cowan 2004). This is why injury in the basal ganglia, thalamus, hippocampus and cerebral cortex is most commonly observed in these situations.

1.1.5. Clinical Severity

Neonatal brain injury is recognized on the basis of a unique encephalopathy that evolves behaviorally from lethargy to hyperexcitability to stupor during the first three days of life (Ferriero 2004, Sarnat & Sarnat 1976). Neonatal brain injury often eludes diagnosis, especially in premature infants with very low birth weight, because obvious signs are lacking or because the signs that are present are attributed to developmental immaturity (Ferriero 2004, Mercuri *et al.* 2003). Commonly, HI infants will initially appear clinically depressed (lethargic with central hypotonia, particularly proximally) and with reduced tendon reflexes (Perlman 2004, Towbin 1970). Infants with these symptoms are often diagnosed with hypoxic-ischemic encephalopathy (HIE). HIE is a neurobehavioral state in which the predominant pathogenic mechanism is impaired cerebral blood flow. The clinical severity of HIE can be graded as mild (grade 1: irritable, hyper-alert, mild hypotonia, poor sucking), moderate (grade 2: lethargy, marked abnormalities of tone, parentral nutrition required), or severe (grade 3: coma, prolonged seizures, severe hypotonia, failure to maintain spontaneous respirations) (Adcock & Papile 2008, Roberson & Finer 1985). Excessive neuronal excitability, development of extrapyramidal neurologic abnormalities, and paroxymal EEG activity are observed in severe HIE. In fact, the severity and degree of neonatal seizures and muscle tone abnormalities post-HI often provide clues as to future outcome. Although there are no absolute predictive measures of outcome post-HI, clinical correlates such as prolonged fetal bradycardia, repetitive late decelerations, low extended Apgar scores, abnormal fetal blood gases, low fetal scalp or cord pH, various abnormal metabolic parameters (ammonia, lactate, CK-BB), and biophysical profiling have been used to identify or verify the severity of HI insult (Sunshine 2003). Markedly, severe fetal acidosis (pH=6.61-6.79) correlates best with encephalopathy and structural-functional damage to other organs.

1.1.6. Outcome After Cerebral Hypoxia-Ischemia

Overall mortality from perinatal HI is 10-30%, with 15-45% of surviving infants sustaining neurodevelopmental sequelae (Adcock & Papile 2008, Roberson & Finer 1985). When specific variables such as encephalopic severity, the presence or absence of seizures, EEG results, and neuroimaging findings are taken into account, the following outcome predictions are ascertained: Grade 1, in which 98-100% of infants will have a normal neurologic outcome and <1% mortality; Grade 2, where 20-37% may die or have abnormal neurodevelopmental outcomes and Grade 3, in which 50-89% die and all survivors have major neurodevelopmental impairment (Adcock & Papile 2008).

1.1.7. Neurologic Sequelae

Regardless of the type or nature, it is well established that cerebral HI is the single most common cause of neurological and intellectual handicaps in children (Sunshine 2003). Many infants that survive HI injury have significant neurologic complications that are diverse in their progression, presentation and contribution to long-term disability. Many psychiatric, neuropsychiatric and neurological disorders such as cerebral palsy, mental retardation, epilepsy, ADHD, hearing and vision loss, and learning disabilities (significant psycho-educational handicaps at school age such as learning difficulties in math and spelling, disturbances of language and speech, and soft neurological signs) are commonly linked to HI events before, during or after birth (Vannucci 2000). Schizophrenia, conduct disorders and developmental diseases such as neuronal migration disorders, lissencephaly and heterotopias, also likely have some degree of cerebral HI linked to their etiology (Johnston *et al.* 2002a, Schubert *et al.* 2005).

1.1.8. Clinical Management of Cerebral Hypoxia-Ischemia

Currently, the post-natal management of cerebral HI is limited solely to supportive therapy. Besides judicious fluid management and seizure control, physiologic metabolic state (glucose, calcium, and magnesium), blood gases and normothermia are monitored and maintained. In addition to the supportive therapy, goals for the management of a HI infant at risk for evolving brain damage should include: 1) early identification of other problems most likely to contribute to injury evolution (i.e.

shock); and 2) consideration of interventions to ameliorate the processes of ongoing brain injury (Perlman 2004, Towbin 1970). There are increasing data to indicate that infants at the highest risk can be identified shortly after birth by a constellation of findings, including: evidence of a sentinel event during labor (fetal heart rate abnormality), severe depression (low extended Apgar score), resuscitation in the delivery room (intubation, chest compression ± epinephrine administration), evidence of severe fetal academia, early abnormal neurologic examination and/or assessment of cerebral function (integrated EEG) (Hellstrom-Westas *et al.* 1995, Perlman 2004, Perlman & Risser 1996, Shalak *et al.* 2003).

Many different strategies for post-HI intervention have been clinically examined. Examples of some interventions that have been investigated in animal and/or human trials include: Interventions to reduce cerebral edema such as mannitol and glucocorticoids, anticonvulsants (midazolam, phenobarbital), calcium channel blockers, magnesium sulfate, γ-aminobutyric acid (GABA) potentiation therapies (topiramate), interventions that reduce free radical damage (allopurinol, N-acetylcysteine, air instead of oxygen for resuscitation), hypothermia, endorphin antagonists, desferoxamine and erythropoietin (Whitelaw 2000, Whitelaw & Thoresen 2002). Despite the numerous studies on the agents listed above, none of the data support routine use of these therapies in neonatal HIE. Of all those listed above, hypothermia has had the most successful transition from the bench to the clinic, although there has been some controversy over the length and depth at which hypothermia should be administered. Despite this, it is well recognized that early induction of hypothermia provides the most effective protection against HI insult. Currently in Edmonton, systemic and local (i.e. head cooling) hypothermia is administered to infants with moderate-severe HIE. It is initiated within 6h of injury and body temperature is maintained at 33-34 °C for 3d (Personal Communication, Po-Yin Cheung, 2008).

There have also been numerous animal and clinical trials investigating the effects and consequences of resuscitating HI babies with room air instead of 100% oxygen. Even though there is international debate on the concentration of oxygen to be used upon initiation of resuscitation, many studies highlight the benefits of room air resuscitation and it well established that there is less injury related to oxidative stress when 21% oxygen is used (Vento *et al.* 2001a, Vento *et al.* 2001b, Vento *et al.* 2005, Saugstad 2004, Saugstad 2005b, Saugstad 2005a, Saugstad *et al.* 2005, Cheung *et al.* 2007, Haase *et al.* 2004, Haase *et al.* 2005).

1.1.9. Pathophysiology

After HI insult, blood flow and oxygen and glucose delivery to the brain cease. This interruption in nutrient delivery is devastating as the brain's high metabolic rate can not be sustained. Further, toxic metabolites such as lactic acid and carbon dioxide accumulate and cellular acidification occurs. Primarily, immediate neuronal cell death occurs due to intracellular osmotic overload (increased Na^+ and Ca^{2+}), as seen with excessive excitatory amino acids acting on ionotropic glutamate receptors and the failure of Na^+/K^+ ATPases. Delayed neuronal cell death occurs secondary to uncontrolled activation of enzymes and second messenger systems within the cell (calcium-dependent lipases, proteases), perturbations in mitochondrial respiration, free radical generation, nitric oxide (NO) generation, induction of inflammation and/or depletion of energy stores.

1.1.10. Immediate Cell Death

When cerebral HI is severe enough to produce irreversible tissue injury, the insult is almost always associated with major perturbations in the energy status of the brain (Vannucci 1990, Vannucci 1993b). During the early course of a HI insult, alterations in adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP), and phosphocreatine (PCr) occur (Vannucci 1990, Vannucci 1993b). This loss of cellular ATP during HI severely compromises those metabolic processes that require energy for their completion. Further, oxidative phosphorylation ceases and anaerobic glycolysis becomes the only source of energy. As the neuronal demand for energy is so high, all reserves are depleted within 2-3 minutes and anaerobic production of ATP cannot sustain brain cells at a functional level (Nestler *et al.* 2001). Consequently, ATP-dependent processes such as Na^+/K^+ exchange is curtailed and there is a resultant intracellular accumulation of Na^+, Cl^- and water (Vannucci 1993a, Vannucci *et al.* 1993). This results in cytotoxic edema occurring from the net movement of osmotically driven water from the extracellular, to the intracellular space (Vexler & Ferriero 2001). Without the regeneration of ATP, endergonic reactions do not resume, intracellular ions and water continue to accumulate, and electrochemical gradients cannot be reestablished. With this, other factors ultimately influence tissue cellular integrity such as the formation of free radicals and the associated peroxidation of free fatty acids within cellular membranes, and cellular acidosis (Dirnagl *et al.* 1999, Vannucci 1993a). As ischemia impairs cellular metabolic rates and depletes the energy required to maintain ionic gradients, neurons and glia lose their membrane potential and depolarize (Katsura *et al.* 1994). These depolarizations allow somatodendritic and presynaptic voltage-dependent calcium channels to become activated, and excitatory amino acids are released into the extracellular space (Dirnagl *et al.* 1999). In addition, the presynaptic re-uptake of neurotransmitters becomes impaired, and amino acids accumulate in the extracellular synaptic cleft.

A fundamental process responsible for a large proportion of early HI cell death is excitotoxicity, or cell death mediated by excessive stimulation of excitatory amino acid receptors (Figure 1.1). Normally these receptors mediate the physiologic excitatory effects of glutamate, one of the most ubiquitous and versatile neurotransmitters in the brain (Johnston *et al.* 2002b). However, when excessively stimulated by combinations of elevated synaptic levels of glutamate and membrane depolarization associated with ischemia, the channels associated with these receptors allow a lethal flood of Ca^{2+} and Na^{+} to enter neurons (Johnston *et al.* 2002b). This activation of glutamate receptors, through the attendant failure of ion homeostasis and increases in intracellular calcium, is a major factor involved in the initiation of ischemic cell death (Dirnagl *et al.* 1999). Downstream consequences of this include activation of lipases, endonucleases, proteases and caspases and degradation of the cytoskeleton and cell membrane.

1.1.11. Delayed Cell Death

Not only does excitotoxicity cause acute cell death, it can also initiate molecular events that lead to delayed or secondary cell death. Excitotoxicity can upregulate signaling pathways that initiate post-ischemic inflammation (Dirnagl *et al.* 1999). Previously, inflammation has been shown to be involved in the pathogenesis of numerous brain diseases including traumatic brain injury, adult cerebral ischemia and neurodegenerative disorders such as Alzheimer's and Parkinson's diseases (McGeer & McGeer 1995, Merrill 1992, Yrjanheikki *et al.* 1999, Barone & Feuerstein 1999, Dirnagl *et al.* 1999). Inflammation is regarded as a promising pharmacological target as it progresses several days after injury (Figure 1.1), and because intervention with anti-inflammatory therapies may not result in intolerable side effects as the wide clinical use of non-steroidal anti-inflammatory drugs demonstrates (Barone & Feuerstein 1999, Tikka *et al.* 2001). Inflammation contributes to brain cell death after HI by launching an immune attack from both the blood and the brain. It is mediated by both molecular components including cytokines and cellular components (i.e. leukocytes and microglia) (Emsley & Tyrrell 2002). Following injury to the CNS, blood-derived macrophages and activated microglial cells accumulate at the site of the lesion (Rabchevsky & Streit 1998). The inflammatory response exacerbates existing vessel and tissue injury and encompasses lipid peroxidation and the extensive production of cytotoxic molecules such as cytokines, chemokines and adhesion molecules.

Cellular initiation of inflammation in the brain by HI injury usually takes place in the microvasculature (del Zoppo *et al.* 2000). Cerebral microvessels are ternary structures consisting of an endothelial cell lining and basal lamina, surrounded by astrocytic end processes (Rosenberg 2002). The microvasculature responds dynamically to flow disturbances, free radical exposure and the presence of cytotoxic molecules (del Zoppo *et al.* 2000). Proteolytic degradation of extracellular matrix components is an important aspect of many physiological and pathological processes, from wound repair and uterine involution to inflammatory diseases and invasive tumors, and plays a major role in the pathophysiology of HI (Kleiner & Stetler-Stevenson 1994). The majority of proteolytic degradation occurring after HI results from the activation of matrix metalloproteinases (MMPs). In their active form, MMPs have a number of important roles in normal development, but they are highly destructive in neuroinflammation (Rosenberg 2002, Yong *et al.* 2001). MMPs degrade the components of the basal lamina, including heparin sulfate, laminin, fibronectin, and type IV collagen (Yong *et al.* 2001, Brundula *et al.* 2002). This proteolysis of the ECM contributes to HI brain tissue damage either by directly attacking the lining of the blood vessel and increasing the permeability, or by loosening the connections between the cells, breaking communication channels for signaling molecules and nutrients (Rosenberg 2002). Thus, vasogenic edema results from disruption of the blood-brain barrier with resultant free passage of large molecular weight molecules, including protein and water, from blood into parenchyma (Dirnagl *et al.* 1999, Vannucci 1993a).

The major inflammatory cells to accumulate and become active within the brain after HI are the blood-borne leukocytes and resident microglia (Emsley & Tyrrell 2002). Microglia are the innate inflammatory and brain-resident tissue macrophages of the CNS (Hanisch 2002, Liu & Hong 2003, Magnus *et al.* 2002, McGeer & McGeer 1995, Streit 2002, Lai & Todd 2006b). Normally, they are present in their resting or ramified form, but when the CNS is injured, they are the first non-neuronal cells to become activated to release cytokines and other neurotoxins, NO, free radicals and MMPs (Emsley & Tyrrell 2002, Rosenberg 2002, Tikka & Koistinaho 2001). The hallmarks of gliosis are proliferation and hypertrophy of astrocytes and cells derived from the mononuclear phagocytic system, including macrophages and microglia (del Zoppo *et al.* 2000). Activated microglia actively retract their cytoplasmic processes and increase the expression of cell surface antigens. They become phagocytic when fully activated and are known to enhance NMDA receptor-mediated neurotoxicity (Tikka & Koistinaho 2001).

Proinflammatory cytokines, including tumor necrosis factor alpha (TNFα) and interleukin 1-beta (IL-1β), are directly neurotoxic and are responsible for the accumulation of inflammatory cells in the injured brain. TNFα is a cytokine with potent stimulatory actions and has a fundamental role in the propagation of inflammation. It has been detected in ischemic brain cells in experimental animals and is believed to play an important role in apoptosis (Sairanen *et al.* 2001). During HI, cytokines also attract leukocytes and stimulate the production of adhesion receptors on these leukocytes and endothelial cells (Barber *et al.* 2001). Leukocytes promote infarction through the immune reaction they mediate directly and through their toxic by-products, phagocytic action, and interactions with microglia (Barber *et al.* 2001). The expression of adhesion receptors, including those for intracellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and endothelial cell adhesion molecule-1 (ECAM-1) are induced and allow for complementary interaction with neutrophil receptors (Okada *et al.* 1994). Neutrophils are then able to adhere to the endothelium, cross the vascular wall and enter into the brain parenchyma (Mori *et al.* 1992). Consequently, macrophages and these neutrophils migrate into the ischemic brain tissue and begin to phagocytose cells. This action further potentiates the inflammatory cascade by releasing more neurotoxic molecules and recruiting more inflammatory cells.

Cell death is the final, common endpoint of the diverse pathways activated in HI. After HI, cell death occurs via a continuum of necrosis and apoptosis. Necrosis is a pathological process in which a death stimulus (i.e. ischemia) is the direct cause of the demise of the cell (Friedlander 2003). Necrosis occurs very quickly and in a disorganized fashion. Acutely after HI or trauma, necrosis occurs in the areas of the brain that are most severely damaged and usually affects a large number of contiguous cells (Love 2003). Histologically, necrosis can be identified by mitochondrial and nuclear swelling, dissolution of organelles and condensation of chromatin around the nucleus (Friedlander 2003). Following rupture of the nuclear and cytoplasmic membranes, deoxyribonucleic acid (DNA) is degraded randomly via multiple enzymatic degradations.

Apoptosis on the other hand, is a very orderly process in which a death stimulus activates a cascade of events that orchestrates the destruction of a cell (Friedlander 2003). It is a fundamentally important process whereby the brain eliminates non-functional cells during development (Wang *et al.* 2001), and its cascade can be activated at any time during the cellular life cycle. Also known as programmed cell death type 1, apoptosis is an energy-dependent process and can be identified by cell shrinkage, chromatin condensation, orderly disintegration of the plasma membrane (membrane blebbing), and nuclear fragmentation (Wyllie *et al.* 1981, Bredesen 1995).

Despite being important for the maintenance of tissue homeostasis and a critical element in development and the removal of cells following exposure to toxic compounds (Ashe & Berry 2003), apoptosis is implicated in the pathophysiology of HI. Brain cells that have been compromised by excessive glutamate receptor activation, calcium overload, free radicals and/or by mitochondrial and DNA damage can die by apoptosis. Apoptosis is underscored not by a series of clearly defined pathways, but rather a multitude of highly regulated, inter-connected pathways (Figure 1.2) (Ashe & Berry 2003). Mammalian apoptosis is regulated through crucial signal transduction via the B-cell lymphoma-2 (Bcl-2) family of proteins, the apoptotic protease-activating factor adapter protein (Apaf-1), and the cysteine protease caspase family (Yuan & Yankner 2000). Depending on the type of neuron and the stage of development, different combinations of Bcl-2 and caspase family members are expressed and provide specificity of regulation in response to HI. The Bcl-2 gene family contains both anti- and pro-apoptotic proteins. The major anti-apoptotic members are Bcl-2 and Bcl-XL and the major pro-apoptotic members are Bax and Bad. Each is localized in the outer mitochondrial membrane and the endoplasmic reticulum in neurons (Yuan & Yankner 2000).

Caspases are also important in the induction of programmed cell death. They are a family of fourteen cysteine proteases that are constitutively present in cells (in their inactive form) and are involved in the initiation and execution of apoptosis (Ashe & Berry 2003). The caspases can be divided into two broad groups: those of the interleukin-1 converting enzyme family (caspase-1, -4, -5, -14) that are involved in the processing of cytokines and the induction of inflammation, and those that are directly involved in apoptosis (-2, -3, -6, -7, -8, -9 and –10) (Earnshaw *et al.* 1999, Strasser *et al.* 2000). The caspases in the latter group can be further subdivided into the initiator caspases (-2, -8, -9, and –10) and the effector caspases (-3, -6, and 7) (Love 2003). The initiator caspases help to transduce pro-apoptotic stimuli into proteolytic activity, while effector caspases are directly responsible for the cleavage of cellular substrates and are accountable for the majority of the morphological and biochemical features of cell death (Kauffmann & Hengartner 2001).

There are two main pathways of caspase activation in a cell: death receptor-mediated and mitochondrial-mediated (Figure 1.2) (Kauffmann & Hengartner 2001, Ashe & Berry 2003). Principal death receptors in mammalian cells include those for Fas and TNFα, death receptor-4 (DR4), and death receptor-5 (DR5) (Strasser *et al.* 2000), which transmit their apoptotic signals following the binding of death ligands. These receptor-ligand complexes initiate apoptotic cascades within seconds of ligand binding and can result in apoptotic cell death within hours (Ashe & Berry 2003). Fas and TNFα bind to their respective receptors and commence a chain of events that usually ends with the activation of caspase-8 and caspase-3. The cleavage of procaspase-3 into its active form (cleaved caspase-3) precedes DNA laddering and formation of apoptotic bodies. The intrinsic cell death pathway involves the initiation of apoptosis as a result of a disturbance in intracellular homeostasis (Ashe & Berry 2003). In this pathway the signals for death are initiated within the cell itself, and mitochondria are crucial for the execution of cell death. This mitochondrial pathway of caspase activation occurs with the release of cytochrome c from damaged mitochondria, and central to this pathway is the aforementioned Bcl-2 family (Ashe & Berry 2003, Morrison *et al.* 1998).

1.1.12. Developmental Cell Death

Pruning, or developmental apoptosis, is required in the neonatal brain to determine the numbers of neurons in subpopulations and to eliminate cells with DNA damage. The neonatal brain is inherently primed for apoptosis as it matures when significant axon outgrowth and synaptic consolidation occur. Further, damaged neurons and abnormal connections are eliminated as any neuron not firing is programmed to die. All cell types in the brain, including glia, neural precursor cells, immature neurons and synapse- bearing neurons are pruned and dysregulation of pruning results in developmental brain pathology.

1.1.13. Neurogenesis

In the neonatal brain, neurogenesis and neuronal migration occur as part of normal development. However, neurogenesis can be affected by the environment, trophic factors, drug treatments and pathophysiological conditions. Not only can neurogenesis be negatively impacted post-HI, neurogenic processes may become upregulated as part of injury response and compensation (Ong *et al.* 2005, Hayashi *et al.* 2005, Bartley *et al.* 2005). Neurogenesis occurs in two major prolifatory regions of the mammalian brain, the dentate gyrus (DG) and the subventricular zone (SVZ). In the DG, newly generated neuronal cells in the subgranular layer migrate to the granular layer and extend axonal projections to *cornu ammonis* 3 (CA3) of the hippocampus (Taupin 2007, von Bohlen und Halbach 2007). Newborn cells in the anterior part of the SVZ migrate through the rostro-migratory stream to the olfactory bulb, where they differentiate into interneurons and granule and periglomerular neurons (Lois & Alvarez-Buylla 1994, Taupin 2007). Accumulating data now indicates that these areas of ongoing neurogenesis proliferate dramatically after brain injury, expanding and contributing to streams of neuroblasts that track toward damaged brain tissue (Lee *et al.* 2006, Arvidsson *et al.* 2002, Parent *et al.* 2002, Zhang *et al.* 2004).

Thymidine analogues, such as 5-bromo-2-deoxyuridine (BrdU), incorporate into DNA of dividing cells during the S-phase of the cell cycle. As such, they are used for birth dating new cells and *monitoring* cell proliferation (Taupin 2007). However, it is important to recognize that BrdU is a marker of DNA synthesis; not a *direct marker* of cell proliferation (Taupin 2007). This is because BrdU also labels cells undergoing DNA repair, abortive cell cycle reentry as a prelude to apoptosis, and gene duplication without cell division (Taupin 2007). A single injection of BrdU labels the nuclei of cells in S-phase, but not the nuclei of cells that are in other phases of the cell cycle (the series of events that take place in eukaryotic cells leading their replication). Once incorporated into the DNA, BrdU can be visualized and detected by immunohistochemistry using an antibody against single-stranded DNA containing BrdU (Gratzner 1982, Taupin 2007). BrdU is visible immunohistologically in postmitotic cells for the remainder of their life, unless the cells undergo consecutive proliferation, which will exponentially dilute the BrdU signal in the nucleus (Ward *et al.* 1991, Cooper-Kuhn & Kuhn 2002).

Because DNA repair involves DNA synthesis and because BrdU is not a marker of cell proliferation but rather a marker of DNA synthesis, there is concern that BrdU immunohistochemistry may not only detect dividing cells in the brain but also cells undergoing DNA repair (Taupin 2007). Cell death normally occurs in neurogenic zones since a significant proportion of newly generated cells undergo apoptosis rather than achieving maturity (Cameron & McKay 2001). Further, a large proportion of cell death occurs after HI in neurogenic zones, although the extent of this phenomenon is thought to be dependent on injury severity. Even though cell cycle reentry is not a common mechanism of neurodegeneration, since most dying neurons do not pass the G1/S-phase checkpoint to resume DNA synthesis, this phenomenon could lead to false positives when using BrdU to study neurogenesis (Snider *et al.* 1999, Taupin 2007). Specifically, HI could trigger terminally differentiated neurons to reenter the cell cycle and incorporate BrdU (Kuan *et al.* 2004). Thus, double labeling for caspase or TUNEL staining in conjunction with BrdU labeling is essential in all studies of neurogenesis to convincingly prove that the cells in question are indeed "new" and not simply re-entering the cell cycle before death.

Newly generated neuronal cells in the brain temporally and spatially express a sequence of markers (Kuhn *et al.* 1996, Palmer *et al.* 2000, Taupin 2007). Depending on the BrdU application (duration, concentration of applied BrdU, and survival times after BrdU injection), the numbers and phenotypes of labeled cells within the brain can vary (Cameron & McKay 2001, von Bohlen und Halbach 2007). BrdU labels all S-phase cells regardless of fate, and thus does not allow newly formed glia and neurons to be distinguished from each other. During the proliferation stage of neurogenesis, newly generated cells express markers for GFAP and nestin (von Bohlen und Halbach 2007), and as neurogenesis progresses through to differentiation and migration, cells lose their GFAP immunoreactivity and become solely nestin-positive (Kronenberg *et al.* 2003). With maturation, cells stop expressing nestin and start to express doublecortin and the polysialylated embryonic form of the neural cell adhesion molecule (PSA-NCAM) (Kronenberg *et al.* 2003, von Bohlen und Halbach 2007). During the late stages of neurogenesis and axonal and dendritic targeting, newly generated cells become post-mitotic (von Bohlen und Halbach 2007). This is when markers such as calretinin, calbindin and neuron specific nuclear protein (NeuN) become detectable in concert with BrdU.

2.0 Doxycycline For The Treatment of Neonatal Hypoxia-Ischemia

Doxycycline (DOXY) is a lipophillic, second-generation tetracycline derivative that has anti-inflammatory actions independent of its anti-microbial actions (Figure 1.3) (Tikka *et al.* 2001, Yrjanheikki *et al.* 1998). DOXY is commonly used in pharmaceutical formulations, under two chemical forms: Doxycycline monohydrate and doxycycline hyclate. DOXY and other newer tetracyclines have a variety of substituted functional groups at various positions that broaden their antibiotic spectrum by increasing lipid solubility. As such, it crosses the blood-brain, blood-cerebral spinal fluid and placental barriers and has excellent brain and body tissue penetration. DOXY is classified as a long acting tetracycline based on a serum half-life of 16-18h in humans. Generally the distribution and penetration of antibiotics in tissue and interstitial fluids depends on free antibiotic concentration in the central compartment, and as a rule, antibiotics that are more than 80% bound to serum proteins have a very low distribution in the body (Michel *et al.* 1984, Chambers 2001). Although DOXY falls into this category as it is very highly protein bound (82-93%), its has extensive tissue distribution (Michel *et al.* 1984). DOXY's high relative lipid solubility readily compensates for high protein binding (Michel *et al.*

1984). Absorption of DOXY after oral administration is 95-100%, and it is not impaired by food in the upper small intestine (Chambers 2001). Even though other tetracyclines are excreted in the bile and urine, DOXY is unique as it is eliminated by non-renal mechanisms, does not accumulate significantly in situations of renal failure, and requires no dosage adjustment in cases of renal insufficiency (Chambers 2001).

As an antimicrobial, DOXY is broad spectrum. It inhibits protein synthesis and is bacteriostatic for many gram-positive and gram-negative bacteria (Chambers 2001, Chopra *et al.* 1992). Similarly to other tetracyclines, DOXY enters microorganisms by passive diffusion and active-transport, and is concentrated intracellularly in susceptible cells. Once inside the cell, DOXY binds reversibly to the 30S ribosomal subunits on bacterial ribosomes, and prevents protein biosynthesis by blocking the binding of amino-acyl-tRNA to the acceptor site on the mRNA-ribosome complex and the addition of amino acids to the peptide chain (Chambers 2001).

1.2.1. Clinical Uses

DOXY is approved for the use in the treatment and prevention of the following infections: otitis media, Chlamydia, Rekettsia, Brucellosis, Lyme disease, human granulocytic ehrlichiosis, anthrax poisoning, pelvic inflammatory disease, prostatitis, nongonococcal urethritis, urinary tract infections, intra-abdominal and surgical infections, Traveler's diarrhea (bacterial gastroenteritis), sinusitis, tonsillitis, cellulitis, abscesses, gangrene, malaria, syphilis, Q fever, helicobacter, vancomycin resistant enterococcus (VRE), methecillin resistant Staphylococcus aureus (MRSA),

and Legionella (Axisa *et al.* 2000, Joshi & Miller 1997). It can also be used as part of combination therapy to treat gastric and duodenal ulcers caused by Helicobacter pylori and in vibrio infections such as cholera (Chambers 2001).

1.2.2. Adverse Reactions and Drug-Drug Interactions Related to Clinical Use of Doxycycline as an Antimicrobial

In terms of bacterial infections, DOXY is usually the tetracycline of choice because it can be given in once-daily preparations, its absorption is not significantly affected by food and because it has a lesser side-effect profile compared to other tetracyclines. However, like all pharmacotherapies, the tetracyclines do have side-effects and patients can have adverse reactions. Rather than hypersensitivity reactions common with other antibiotics (drug fever, skin rashes, anaphylaxis), most of DOXY's adverse effects are due to direct toxicity or to alteration of innate microbial flora (Chambers 2001). Nausea, vomiting and diarrhea are the most commonly reported reasons for discontinuing the use of tetracyclines and these effects are easily attributable to direct irritation of the gastrointestinal tract. Hepatotoxicity, renal toxicity, venous thrombosis, photosensitization and vestibular reactions have also been reported. Dizziness, vertigo, nausea and vomiting are most commonly associated with doses of DOXY above 100mg (Chambers 2001). Also when used at doses of 100mg and higher, pseudo-tumor cerebri, intercranial hypertension, severe headache, diplopia, and other ocular problems such as loss in visual acuity, visual obscurations, and visual field loss have been reported (Lockhead & Elston 2003, Friedman *et al.* 2004).

As multi-drug regimens and polypharmacy are commonly encountered, the evaluation of DOXY with concomitant administration of other drugs has been investigated. Significant drug-drug interactions have been reported when DOXY is administered with oral antacids and iron-containing preparations, anticonvulsants (carbamazepine and diphenylhydantoin), barbiturates, ethanol, oral contraceptives and penicillin. With the exception of the oral antacids which are chelated by DOXY, causing insoluble salts to be formed and incomplete absorption, the other drugs mentioned significantly decrease DOXY's half-life. Like most other antibiotics, DOXY reduces conjugated estrogen levels and thus, interacts with oral contraceptives.

Even though the aforementioned adverse reactions and contraindications have been reported when DOXY has been used as an antibiotic, the information provided is vital for investigations of DOXY in other settings. DOXY has been used for many years and has a well established safety profile. Further, it is the only tetracycline approved for use in the treatment of complicated infections in the pediatric population. Compared to other drugs in its class and other pharmacotherapies investigated for the putative treatment of neonatal HI, DOXY has a lesser side-effect profile.

1.2.3. Drug-Drug Interactions and Contraindications Specific to the Pediatric Population

All tetracyclines, as a result of their ability to chelate calcium, can bind and damage growing bones and teeth. When DOXY is given during pregnancy, it may be deposited in the fetal teeth, leading to fluorescence, discoloration, and enamel dysplasia (Chambers 2001). To avoid this deposition, tetracyclines are generally avoided in pregnant women and for children under 8-years of age. The formation of fetal teeth occurs in the 6-24th week of pregnancy. Primary tooth formation and development usually begins between the 13-16th weeks of gestation for the incisors and between the 14-24th weeks for canines and molars (Billings *et al.* 2004). Mineralization of the enamel surface is usually complete by the first postnatal year. Permanent dentition begins to form *in utero* and mineralization is usually complete by age 4-5 (Billings *et al.* 2004). Teeth are most susceptible to developmental disturbance during the mineralization phase of tooth formation, and permanent dentition is more likely to be affected by environmental toxins and drugs than is the primary dentition (Billings *et al.* 2004). Thus, the likelihood of DOXY affecting the development of fetal teeth is highly unlikely. This therapy, if approved for use in neonatal HI brain injury, would most likely be administered after the 20th week of pregnancy since the risk of significant birth trauma and complications increases as the fetus ages. Further, treatment with DOXY during periods of enamel calcification either by a mother during gestation or a small child does not significantly stain teeth at all (Billings *et al.* 2004).

1.2.4. Special Considerations in Pediatric Pharmacology

Although general pharmacologic principles apply to patients of all ages, the physiologic context in which these principles operate are different in pregnant women and infants (Koren 2001). Critical factors affecting placental drug transfer and drug effects on the developing fetus include: 1) the physiochemical properties of the drug; 2) the rate at which the drug crosses the placenta and the amount of drug reaching the fetus; 3) the duration of exposure to the drug; 4) distribution characteristics in fetal tissue; 5) the stage of placental and fetal development at the time of exposure to the drug; and 6) the effects of drugs used in combination (Koren 2001). Drugs that cross the placenta enter the fetal circulation via the umbilical vein. As DOXY is able to cross the placenta and there is no contraindication for DOXY treatment in the fetus, it may be administered to expectant mothers (Czeizel & Rockenbauer 1997).

Drug therapy in infants and children requires special attention to differential drug absorption (blood flow at site of administration, gastrointestinal function), distribution, metabolism and excretion, as pharmacokinetic and pharmacodynamic variables in the infant change significantly in the first few months of life (Koren 2001, Besunder *et al.* 1988b, Besunder *et al.* 1988a). As newborns generally have less muscle mass and more water content than adults, the distribution volumes of drugs changes significantly. Differences may also be observed between full-term neonates (70% body weight as water) and low birth weight premature infants (85% body weight as water) (Koren 2001). Further, preterm infants have significantly less fat than term infants and thus, organs that generally accumulate high concentrations of lipid-soluble drugs in adults and older children may accumulate smaller amounts in premature infants (Koren 2001). Further, protein binding of drugs is often reduced in neonates on account of the lower glomerular filtration rates that exist in more immature kidneys.

1.2.5. Tetracyclines and Neuroprotection

In addition to their antibiotic properties, tetracycline and its derivatives, doxycycline and minocycline (MINO), have anti-inflammatory and anti-apoptotic properties (Sanchez Mejia *et al.* 2001, Amin *et al.* 1996, Chen *et al.* 2000). DOXY and MINO were previously reported to be neuroprotective in global and focal brain ischemia in adult animals, as well as in culture models of cell death (Tikka *et al.* 2001, Yrjanheikki *et al.* 1998, Yrjanheikki *et al.* 1999). These drugs significantly protect hippocampal neurons from global and focal ischemia (Yrjanheikki *et al.* 1998) and delay mortality associated with other types of brain injury such as Huntington's disease (Chen *et al.* 2000). Further, MINO has been reported to be neuroprotective in neonatal animals (Arvin *et al.* 2002). Both DOXY and MINO have direct effects on microglia (Lai & Todd 2006a) and are capable of modulating ATP receptors as they have been found to decrease numbers of P2YR2 (Lai *et al.*, 2008, unpublished observations). In addition, they have been shown to inhibit mediators of inflammation such as inducible nitric oxide synthase (iNOS), NO, cyclooxgenase-2 (COX-2) and many cytokines. They also inhibit tumor-induced angiogenesis, malignant cell growth, bone resorbtion, and the release of reactive oxygen species from polymorphonuclear cells (PMNs). These members of the tetracycline family are potent MMP inhibitors, due most probably to their ability to chelate Zn^{2+} from the active site of the MMPs (Golub *et al.* 1991, Duivenvoorden *et al.* 2002). It has been reported that DOXY is effective in decreasing stimulated cerebral MMP-9 activity and parenchymal angiogenesis (Lee *et al.* 2004).

MINO, the most well known and more widely experimentally tested second generation tetracycline, has many documented mechanisms of action. It is thought to be an attractive pharmacological agent in disorders of neuroinflammation and neurodegeneration because of its oral bioavailability, ability to cross the blood-brain barrier, and it extensive use in humans for a variety of acute and chronic conditions (Sanchez Mejia *et al.* 2001). It has been proven to be efficacious in a variety of experimental models of neurologic diseases, with its beneficial effects thought to be a result of its ability to inhibit the induction of interleukin 1β converting enzyme (ICE), prevent iNOS protein expression, inhibit caspase production, decrease the expression of COX-2 and prevent the activation of microglia (Chen *et al.* 2000, Tikka *et al.* 2001, Yrjanheikki *et al.* 1998, Yrjanheikki *et al.* 1999). Further it has been shown to suppress BAX accumulation, and induce Bcl-2 (Wang *et al.* 2004). In a mouse model of amyotrophic lateral sclerosis, MINO has been reported to inhibit cytochrome c release and slow disease progression (Zhu *et al.* 2002). In models of Huntington's disease, it has been reported to inhibit both caspase-independent and dependent mitochondrial cell death pathways (Wang *et al.* 2003). In experimental models of traumatic brain injury, MINO is effective in reducing tissue injury and neurological deficits that purportedly occur through caspase-1-dependent mechanisms and a reduction in IL-1β production (Sanchez Mejia *et al.* 2001). MINO has also been shown to protect against cerebral ischemia by reducing infarct volume after focal embolic ischemia in rats (Wang *et al.* 2002), reducing microglial activation, and inhibiting the induction of ICE mRNA (Barone & Feuerstein 1999, Tikka *et al.* 2001, Yrjanheikki *et al.* 1998, Yrjanheikki *et al.* 1999). Further, it has been hypothesized that MINO may function by reducing the cytotoxic properties of microglia, triggered by either ischemia or excitotoxicity, and that excitotoxin-induced activation of p38 mitogen activated protein kinase (MAPK) in microglia is prevented by MINO (Tikka *et al.* 2001). *In vitro*, MINO is capable of attenuating hydrogen peroxide-induced neurotoxicity and may be antioxidative (Lin *et al.* 2003).

1.3. Modeling Neonatal Hypoxia-Ischemia

To study neonatal hypoxia-ischemia, we used an animal model that is globally accepted and widely characterized. Originally developed in 1981, the Vannucci preparation is the gold standard *in vivo* model for studying the short- and long-term effects of cerebral HI on motor activity, behavior, seizure incidence, brain plasticity, and developmental maturation. It has also been widely used to characterize the chronology of pathophysiology and mechanisms of brain damage post-HI and therapeutic intervention in relation to perinatal cerebral HI. Adapted from the Levine preparation of the adult rat (Levine 1960), in the Vannucci model, HI brain damage is induced in post-natal-day 7 (P7) rats subject to permanent unilateral common carotid ligation followed by inhalation of 8% oxygen/balanced nitrogen to produce systemic hypoxia (Vannucci & Vannucci 2005, Rice *et al.* 1981). Throughout the duration of both hypoxia and ischemia the rats are held at a constant temperature (i.e. 33-37°C). This model was originally developed in the P7 rat as it is at this stage the rat's brain is histologically and developmentally similar to that of a 32- to 34-week gestation human fetus (cerebral cortical neuronal layering is complete, the germinal matrix is involuting and little myelination has occurred), although it has been applied to newborn rodents at all stages of development (Vannucci *et al.* 1999, Vannucci & Vannucci 2005). Depending on time spent in hypoxia, injury severity can be titrated from milder forms of cerebral HI (patchy neuronal cell loss, glial activation and hypertrophy in defined regions) to moderate-severe presentations of the injury as indicated by significant cerebral atrophy, cystic lesions, significant cortical loss and thinning, ventriculomegaly, volume reduction, hippocampal compression and widespread glial activation. In most experimental protocols, the typical time spent in hypoxia ranges from 45min to 1.5h, although the rat pups are capable of surviving for 3 or more hours before appreciable mortality occurs. In this model, a combination of both hypoxia and ischemia is required to observe significant differences in CBF, and measurements of systemic variables reveal hypoxemia combined with hypocapnia produced by hyperventilation (Vannucci & Vannucci 2005, Welsh *et al.* 1982). Earlier investigations revealed that hypocapnia compensates for metabolic acidosis caused by lactic academia, such that systemic pH does not change from control (Vannucci & Vannucci 2005).

In this HI model, brain damage is observed within hours after insult. Typically, damage is largely restricted to the hemisphere ipsilateral to carotid ligation, and can easily be identified in the cerebral cortex, subcortical and periventricular white matter, striatum, hippocampus and thalamus (Vannucci & Vannucci 2005, Rice *et al.* 1981, Towfighi *et al.* 1995). Cortical damage is often laminar, with layers 3 and 5 and 6 being especially vulnerable, although significant cortical damage is also observed as columns of dead neurons (ghosts) adjacent to columns of preserved neurons oriented at right angles to the pial surface (Vannucci & Vannucci 2005). Perfusion deficits corresponding with this pattern of damage in the cortex, as well as in the dorsal lateral striatum, have been well documented, but it is also widely accepted that metabolic factors and intrinsic vulnerability of certain cell types are responsible for the pattern and distributions of observed brain lesions.

Although it has been used for upwards of 27 years, new investigations are continually undertaken to find ways to reduce variability, enhance likelihood of successful clinical translation and improve this animal model in general. Presently, it is known that the species of rat used in this preparation is important in relation to major developmental milestones. It is now believed that a P7 Sprague Dawley rat has a brain that is developmentally, roughly equivalent to that of a term infant, whereas P6/7 in a Wistar rat corresponds with a 32-34 week gestation fetus and a P10 is more closely related to term gestation. In addition, as more laboratories have used this method it has become clear that there can be significant variability in the severity of brain damage observed in pups who have spent the same length of time in hypoxia. Methods to reduce variation in this preparation include: 1) ensure the body temperature of all animals is the same throughout HI and after, as individual differences in core temperature can alter the extent of brain injury; 2) keep the recovery time in between ischemia and hypoxia constant for all animals as intervals shorter than 2 hours may not allow all pups sufficient suckling time and anything upwards of 4 hours may result in less severe brain damage; and 3) try to hold litter size constant at 10 (Vannucci & Vannucci 2005).

Other models of HI that combine vascular occlusion with hypoxia in non-murine species include those carried out in the Rhesus monkey, 119-133 gestation sheep, P1 rabbit, P14 dog and term piglet. The comparison between these models has demonstrated that the more immature brain appears to have greater *total* resistance to the development of HI injury (Roland & Hill 1997). Thus, species that are less mature at birth (i.e. dog, cat, rat) appear to be more tolerant of HI than those that are more mature (i.e. guinea pig) and often require longer exposures to hypoxia. Although functional deficits do not always correlate well with the often severe cellular damage observed, all are excellent for understanding pathological mechanisms following HI. Piglet models usually combine hypoxia with either transient bilateral common carotid artery occlusion or permanent unilateral common carotid artery occlusion. Like rats, the severity of injury and survival post-HI in piglets depends on the duration of hypoxia, but unlike rodents it also depends on the presence of respiratory support. All piglet models use anesthesia, mechanical ventilation and intensive care measures to promote animal survival after the HI insult. Hypoxia-ischemia can also be modeled in the baboon, P3-P7 rat, 93-96 gestation day sheep and 20-28 day gestation rabbit to investigate cerebral injury common in premature infants and white matter damage. Within these species, damage may be achieved with *in utero* or perinatal infection/endotoxin, excitotoxic injury, chronic hypoxia or HI with vascular occlusion and hypoxia.

1.3.1. Clinical Relevancy of Animal Models

Although there are many differences between rats and primates with respect to the consequences of and responses to HI, there are increasingly more recognized similarities. In humans, severity and duration of hypoxia, type of insult, gestational age and metabolic status including temperature are all important variables that determine pattern of injury and outcome post-HI. Even though the time course of cellular responses to injury in the human fetal and neonatal brain may be different than that observed in the rodent, the same histopathological changes are observed in both species, including edema, coagulation necrosis, axonal swelling, necrotic and apoptotic cell death, swollen endothelial cells, capillary proliferation, microglial activation, cyst formation, reactive astrocytosis, and fibrillary gliosis (Squier & Cowan 2004).

4.0 Objectives of the Present Thesis

As outlined above, neonatal HI is an exceedingly complex medical problem that results in significant cellular, tissue, organ and systemic dysfunction in an affected neonate. Additionally, the brain damage that ensues post-HI is often devastating and leads to life-long disability. With the exception of supportive care, there are no approved treatments for the brain injuries sustained in hypoxic-ischemic infants. However, multiple lines of evidence suggest that inflammation, because of its delayed progression and sustained evolution, may be an appropriate therapeutic target. Given these data, the first objective of this thesis was to investigate the role of microglial activation in relation to the timecourse of neuroinflammation post-HI, and improve the understanding of microglial activation in the context of neuronal cell survival and neuronal cell death in the developing brain. Doxycycline was used as a pharmacological tool to manipulate inflammation post-HI and thus, the second objective of this thesis was to study the putative neuroprotective and neurodevelopmental consequences of DOXY administration following neonatal HI.

To address these objectives the following questions were specifically addressed:

1) Is DOXY able to penetrate the blood-brain barrier in quantifiable amounts? (Chapter 2)

2) What are the acute cellular and amino acid changes observable following HI? (Chapter 2)

3) Are there any acute neurochemical, neuroprotective, anti-apoptotic and/or anti-inflammatory effects associated with DOXY administration in neonatal HI? (Chapter 2)

4) How robust are the neuroprotective properties of DOXY and what are potential mechanisms of action? (Chapter 3)

5) What are the developmental consequences of DOXY administration in concert with HI brain injury as specifically related to cell genesis and neurogenesis? (Chapter 4)

6) What are the potential mechanisms of action for the developmental changes observed after neonatal HI and in concert with DOXY administration, and how do these potentially relate to cell survival and cell death post-HI? (Chapter 5)

Putative answers to these questions and the results of these studies are presented in the following series of manuscripts.

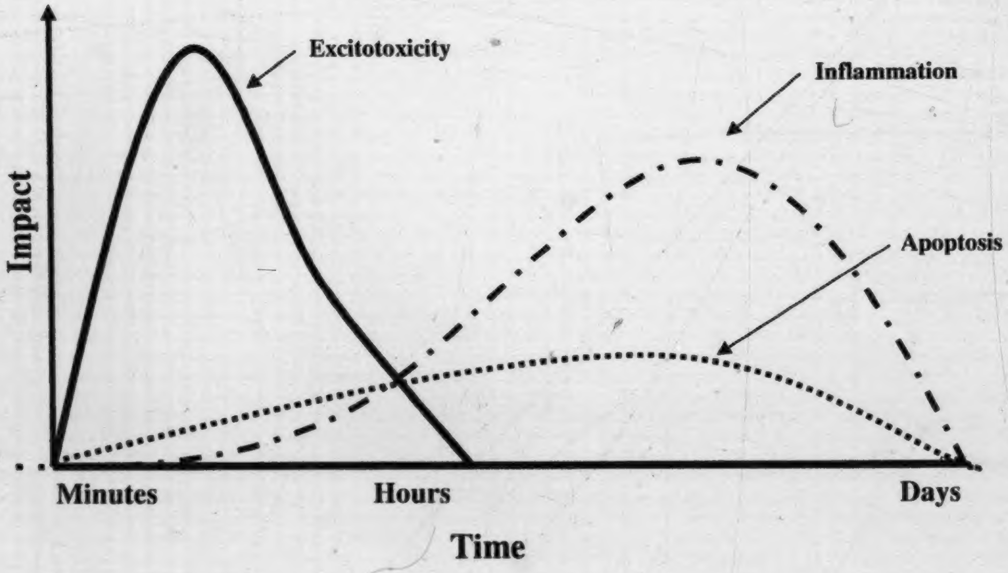
Excitotoxicity
Inflammation
Apoptosis
Impact
Minutes
Hours
Days
Time

Figure 1.1. Temporal Evolution of Major Pathophysiological Events After Neonatal Hypoxia-Ischemia (HI). Immediately following a cerebral HI event, excitotoxicity and peri-infarct depolarizations contribute to cell death related to energy failure, disruption of ion gradients and glutamate receptor activation. Hours-days post-HI, inflammation and apoptosis play larger roles in the evolution of cell death and injury. (Figure adapted from Dirnagl *et al.*, 1999)

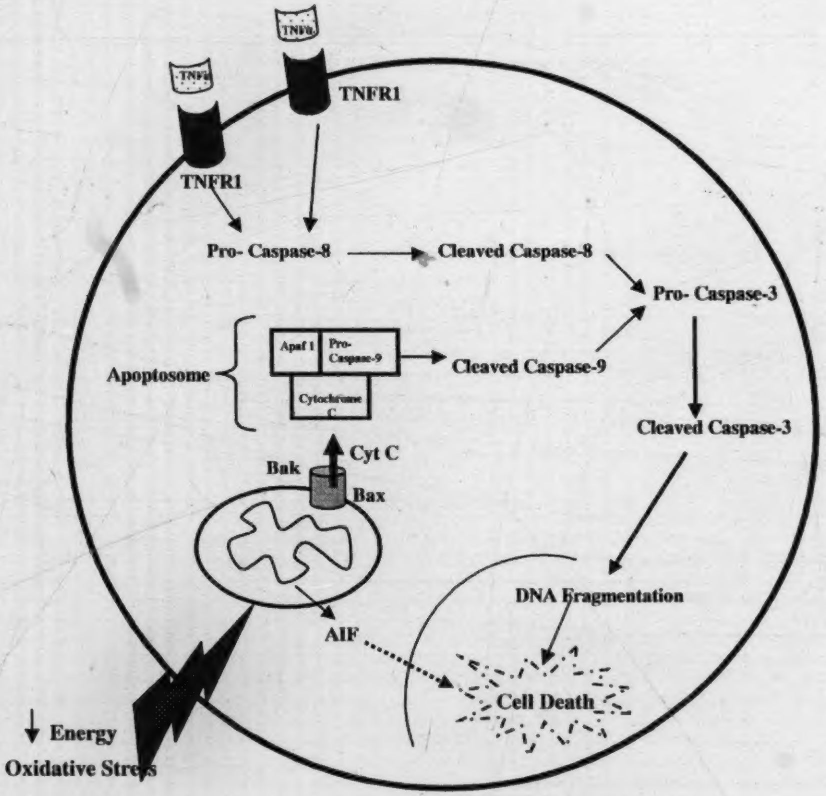
TNFα
TNFα
TNFR1
TNFR1
Pro- Caspase-8
Cleaved Caspase-8
Pro- Caspase-3
Apoptosome
Apaf 1
Pro-Caspase-9
Cytochrome C
Cleaved Caspase-9
Cleaved Caspase-3
Cyt C
Bak
Bax
AIF
DNA Fragmentation
Cell Death
↓ Energy
Oxidative Stress

Figure 1.2. Induction of Apoptotic Cell Death After Hypoxia-Ischemia (HI). The intrinsic apoptotic pathway is driven by formation of oligomeric channels composed of Bax or Bak and the permeability transition pore. These channels permit the release of cytochrome c that subsequently participates in the formation of the apoptosome. Alternatively, the extrinsic pathway is driven by activation of death receptors, such as TNFα, and the cleavage of caspase-8. Both the intrinsic and extrinsic pathways converge on caspase-3, ultimately leading to cell death. The release of AIF from mitochondria leads to apoptotic cell death via caspase-independent mechanisms. (Apaf - apoptotic protease activating factor; AIF - apoptosis-inducing factor; diagram adapted from Calvert and Zhang, 2005 and Northington 2005)

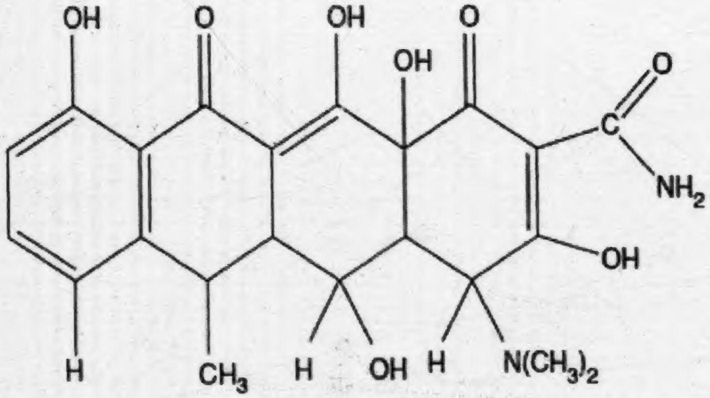
OH
O
OH
OH
O
O
C
NH2
OH
H
CH3
H
OH
H
N(CH3)2

Figure 1.3. Chemical Structure of Doxycycline (DOXY).

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CHAPTER 2

The Acute Neurochemical and Cellular Effects of Doxycycline Administration in Neonatal Hypoxia-Ischemia

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2.1 Introduction

Pregnancy, labour and delivery are dangerous for both mothers and infants, and birth related events that harm the developing brain and subsequent maturation are relatively common (Johnston *et al.* 2002). Disorders of brain development such as mental retardation, and motor system injuries including cerebral palsy, as well as hearing and vision loss, are often associated with lack of oxygen, disruption in the maternal-placental-fetal system, or birth trauma (Little 1862, Johnston *et al.* 2002). In fact, the etiologies of many neurological and psychiatric disorders have been linked to hypoxia-ischemia (HI) and brain injury in early life (Robertson & Finer 1985). Hypoxic-ischemic brain injury is extremely complex. It is diverse in its progression, pathogenesis, and presentation, and it is increasingly evident that therapies are needed to prevent or reduce neuronal cell death associated with the injury. In addition, extensive investigation of the biochemical processes involved in HI is necessary (Roldan *et al.* 1999). The most important aspect of HI brain injury that makes it so difficult to understand and to treat, is that this is an injury that occurs on the forefront of development and on a background of natural, necessary programmed cell death. As such, special patterns of vulnerability are created in the immature central nervous system (CNS) as pathological cell death is superimposed on specific developmental and maturational events (Johnston 1995).

Amino acids have a wide variety of physiological and potentially pathophysiological roles during CNS development. In addition to some being neurotransmitters, many amino acids are involved in intermediary metabolism, the wiring of neuronal networks, building cytoarchitecture, and all are vital to protein synthesis (Ben-Ari *et al.* 1997, Cooper *et al.* 2003, Herlenius & Lagercrantz 2001). This multi-purpose nature makes it difficult to separate their biochemical roles from their neurotransmitter roles, and also makes it hard to completely understand and discern their function in neuronal signal transduction and injury response. In particular, the excitatory amino acids (glutamate, aspartate) participate in signal transduction and exert trophic influences that affect neuronal survival, growth, and differentiation during restricted developmental periods (McDonald & Johnston 1990, Herlenius & Lagercrantz 2001). Because these neurotransmitters are not strictly important for neural trafficking and are vital for the development of neuronal circuits, prenatal or neonatal stress like hypoxia and exposure to certain drugs, may disturb wiring and cause long term neurological and behavioral effects (Herlenius & Lagercrantz 2001).

DOXY is a lipophillic, second-generation tetracycline derivative that has anti-inflammatory actions independent of its anti-microbial actions (Tikka *et al.* 2001, Yrjanheikki *et al.* 1998). Compared to other drugs in its class (Minocycline), DOXY has a lesser side effect profile and is already approved for use in neonates. As it is known that microglia activated after HI and other types of neurodegeneration release neurotransmitters such as glutamate, it is plausible that treatment with anti-inflammatory drugs change the activation state of microglia and thus, the amino acid balance in the brain. Therefore, the goal of the study presented in this chapter was to investigate the effect of DOXY administration on amino acids after mild HI and examine the effects of its administration on acute neuronal cell survival, cellular degeneration, microglial activation and cleaved caspase-3 protein expression.

2.2 Materials and Methods

2.2.1. Chemicals

Doxycycline hydrochloride was purchased from Sigma-Aldrich (St. Louis, MO). The derivatizing reagent *o*-phthaldialdehyde (OPA) was obtained as Fluoraldehyde reagent® from Pierce Chemicals (Rockford, IL). All other chemicals required for high performance liquid chromatography (HPLC) were purchased from Sigma (St. Louis, MO).

2.2.2. Animals and Surgical Procedures

All experiments were performed in accordance with the guidelines set forth by the Canadian Council on Animal Care and were approved by the Health Sciences Animal Policy and Welfare Committee of University of Alberta. On post-natal day 7 (P7), Sprague Dawley rat pups of both sexes, with weights between 12-17 g were anaesthetized with a mixture of isofluorane (4.5% induction, 1.5% maintenance) and oxygen balanced with nitrogen. We used the well-characterized modified-Levine procedure in which unilateral common carotid artery ligation is followed by exposure to hypoxia (Levine 1960, Nakajima *et al.* 2000, Rice *et al.* 1981, Vannucci *et al.* 1999). The modified Levine's procedure was chosen for these investigations because the P7 rat brain is histologically similar to that of the 32-34 week gestation human fetus and because this model produces a reproducible pattern of brain injury that is very similar to the brain damage observed in the human infant (Cheng *et al.* 1998, Vannucci *et al.* 1999). On the anterior ventral surface, a small lateral incision was made at the base of the neck and the right common carotid artery (CCA) was exposed, isolated and permanently ligated. SHAM operated controls underwent the same surgical procedure with the exception of the ligation of the CCA. The wound edges were then opposed and sutured with 4-0 surgical thread and the pups were returned to their dams. The entire surgical procedure lasted approximately 10 minutes. After a two-hour recovery period, the pups were placed in a humidified hypoxia chamber (premixed 8% O_2 balanced with N_2) for 1h. Normothermia was maintained in the chamber at 37.5 °C. Those pups serving as ischemia-only controls were always excluded from the hypoxia chamber and likewise, those pups serving as hypoxia-only controls never received a CCA ligation. Following the hypoxic period, the pups were returned to their dam until euthanasia.

2.2.3. Drug Administration and Tissue Preparation

Rat pups were randomly assigned to treatment groups or to one of the control groups that included SHAM operated controls (common carotid artery isolated, but not ligated) treated with saline vehicle, SHAM operated controls treated with DOXY and, naïve double control animals (no incision, no isolation of the common carotid artery, no DOXY or saline vehicle). Based on previous dose-response studies performed in our laboratory (data not shown) and previous literature reports on the use of doxycycline in adult cerebral ischemia (Yrjanheikki *et al.* 1998), doxycycline hydrochloride (SIGMA, 10 mg/kg) or saline vehicle (VEH) was administered intraperitoneally in a volume of 100 µl as a one-time dose immediately before hypoxia (n≥6). This dose of DOXY is below, but approaches, the lowest end of dosing commonly used for anti-microbial effects in children. Injections were performed using a 3/10cc syringe with a 29-gauge needle (Kendal). Pups were euthanized by decapitation 30 minutes, 1, 2, or 4 hours after the HI insult, as these time points are the most relevant to significant neurochemical changes and acute cellular degeneration. Extracted brains were immediately dissected into frontal-parietal cortex, striatum and hippocampus from both the right and left hemispheres (i.e. hemispheres ipsilateral and contralateral to carotid artery ligation). Dissected regions were stored at –80°C until the time of analyses.

2.2.4. High Performance Liquid Chromatography (HPLC): Doxycycline Extraction

To determine levels of DOXY in the brains of HI pups, remaining portions of brains were weighed and homogenized in ice-cold 0.1N perchloric acid (1:5; w:v). Homogenization in perchloric acid resulted in the best recovery of DOXY as compared to homogenization in ddH_20, mobile phase mixtures or methanol. After homogenization, samples were centrifuged at 4 °C and 9000 xg. The supernatant was collected and loaded into HPLC vials. This method was found to have comparable, if not better, recovery of DOXY from brain (≥40%) when compared to solid phase extraction using an Oasis HLB 1CC extraction protocol (data not shown).

The DOXY extraction assays were performed on a Waters 2695 Separations Module HPLC instrument with a built in vacuum degasser, using a Symmetry Shield RP8

(2.1x150mm) column held at 30 °C. Symmetry Shield guard columns were also used. The composition of the mobile phases were 50/50 methanol/acetonitrile with 0.1% triethylamine (TEA) (mobile phase A) and 10mM NaH_2PO_4 (pH 6.8; mobile phase B). Flow was maintained at 0.5 ml/min and gradients were set at 40% mobile phase A and 60% mobile phase B. A Waters 2487 UV detector was used and UV absorbance was set to 350nm.

2.2.5. Histology and Immunohistochemistry

As doxycycline is an anti-inflammatory compound with anti-apoptotic properties, we also investigated changes in neuronal degeneration and neuroinflammation. A parallel histological and biochemical study was run such that a separate group of pups were used and all pups were subjected to HI, drug treatments and experimental end-points as mentioned above. We wanted to confirm the severity of the HI injury induced and confirm that DOXY was affecting microglia at these time points (30 minutes, 1, 2, or 4 hours post-HI), as glia are significantly involved in release and reuptake of neurotransmitters. Upon removal from the skull, the brains were extracted in their entirety and flash frozen in isopentane. They were then stored at –80°C until serial coronal sections (20 µm) were obtained using a cryostat.

Immunostaining was performed using antibodies recognizing neurons (mouse anti-neuronal nuclei [NeuN], Chemicon, 1:1000), cleaved caspase-3 (rabbit anti-cleaved caspase-3, Cell Signaling Technology, 1:1000), and activated microglia (mouse anti-rat ED-1, Serotec, 1:100). For each animal, immediately adjacent sections were used for each of the primary antibodies.

Fluoro-jade staining was performed to identify dying cells after HI. Specifically, sections were fixed in formalin, washed with water, and dehydrated in a series of ethanol washes. Sections were then incubated in a 0.06% potassium permanganate solution for 15 minutes to suppress background staining. Sections were then washed with ddH_20 and were incubated in a 0.001% Fluoro-jade solution made in 0.1% acetic acid, in the dark for 30 minutes. After washing with ddH_2O again, the sections were placed in a drying oven held at 37.5 °C until completely dry (30-45 min). Sections were then placed in xylene (3x 1 min), and coverslipped with DPX (Fluka).

2.2.6. Western Blotting

To investigate whether caspase-3 and cleaved caspase-3 protein expression was elevated after HI and the acute response to DOXY administration, Western blotting was also performed. These pups received hypoxia and ischemia, and their brains dissected as mentioned above. At the time of analysis, the regional samples were homogenized in 100µl of ddH_2O and 30ul aliquots were taken and diluted with an equal volume of 2x RIPA buffer (2% Igepal CA-630, 1% sodium deoxycholate, 0.2% SDS in PBS) that contained protease inhibitors (complete mini, Roche Biochemical). Fifty micrograms of protein was then loaded onto a 12% SDS-PAGE gel. After electrophoresis, the gel was wet-transferred onto a PVDF membrane at 4°C. The blots were subsequently blocked and incubated with the primary antibody (caspase-3 or cleaved caspase-3 specific, 1:1000) overnight at 4 °C. Target proteins were detected by electrochemiluminescence (ECL). Membranes were re-probed with anti-ß-actin antibody (42 kDa) to verify the consistency of protein loading between lanes.

2.2.7. High Performance Liquid Chromatography (HPLC): Amino Acids

To investigate changes in amino acids in the cortex, striatum and hippocampus, regional samples were hand homogenized in 100µl of ice-cold double distilled water and were then aliquoted. Twenty-five microlitres of homogenate was added to 100µl of ice-cold methanol, rehomogenized and incubated on ice for 10 minutes, and then centrifuged for 4 minutes at 9000 xg and 4°C. Twenty-five microlitres of supernatant was then added to 100µl of ddH_20, and 100µl of this solution was transferred into HPLC vials.

HPLC analyses for the amino acids aspartate (ASP), glutamate (GLU), glutamine (GLN), asparagine (ASN), glycine (GLY), taurine (TAUR), alanine (ALA), serine (SER) and γ-aminobutyric acid (GABA) were performed according to the HPLC principles, methods and set-up used by Parent *et al.* (2001) and Todd & Butterworth (2001). For our assays we used a Waters 2695 separations module with a built in vacuum degasser and a Waters Novapak column (C18 5µm, 3.9x150mm) held at 30°C. In addition, Waters µBondapak guard columns were used and pre-column derivatization with OPA occurred prior to fluorescence detection. Fluorescence emitted by the thioalkyl derivatives was detected using a Shimadzu RF10A fluorescence detector (excitation wavelength 260 nm and emission wavelength 455 nm) following elution of the derivatized amino acids from the column. Additionally, mobile phase composition and mixtures were optimized and a gradient was established. Mobile phase "A" consisted of 900ml 0.08 M NaH_2PO_4 solution, 240ml methanol, 20ml acetonitrile, 10ml tetrahydofuran (THF). This solution was adjusted to pH 6.2 with 10N NaOH, and filtered with 0.2μm pore filters. The composition of mobile phase B was 1340ml 0.04M NaH_2PO_4, 1110ml MeOH, 60ml THF. As above, this solution was also adjusted to pH to 6.2 with 10N NaOH and filtered with 0.2μm pore filters. The mobile phase gradient was set at 95%A and 5%B and flow was maintained at 0.5ml/min. Mobile phase B increased to 100% over 13 minutes. Runtimes were 40 minutes with all compounds eluting by 21 minutes.

Protein amounts in the homogenate were assessed using a bicinchoninic acid protein assay (Sigma), in which bovine serum albumin was used as a standard. Amino acid levels were normalized to the protein amounts as determined by the assays, and amino acid concentrations were expressed as ng/μg protein.

2.2.8. Statistical Analysis

All results shown are expressed as mean ± standard deviation. Data were analyzed by one-way ANOVA followed by the Newman-Keuls multiple comparison test on significant main effects and interactions. Where appropriate, unpaired t-tests were used and when variances between two groups were significantly different a Welch's correction was employed. The general convention of a probability value of $p<0.05$ was used to establish statistical significance.

2.3. Results

Results from this study indicate that systemic administration of DOXY results in global brain distribution. Consistent with a mild HI injury of this nature, significant increases in cleaved caspase-3 protein expression and microglial activation were observed, and neuronal degeneration was seen in the dorsal lateral cortex, striatum, hippocampus, and thalamus, ipsilateral to common carotid artery ligation. In addition, hypoxia-ischemia causes significant acute, time-dependent, regional changes in all brain amino acids examined including glutamate, γ-aminobutyric acid, alanine, aspartate, asparagine, serine, glutamine, glycine and taurine.

2.3.1. Extraction of Doxycycline From Hypoxic-Ischemic Brains

Several reports attest to the ability of second-generation tetracycline derivatives to penetrate the blood-brain barrier. To confirm that DOXY did cross the blood-brain barrier of our HI rat pups, and to analyze the brain levels of DOXY, an extraction assay was performed using high performance liquid chromatography (Figure 2.1). DOXY (10 mg/kg) was administered intraperitoneally as a one-time dose immediately before hypoxia and tissue was collected 1, 2, or 4 hours after the HI insult (total time after DOXY administration = 3.5, 4.5 and 6.5 hours). Results of the HPLC extractions indicate that an average level of 867.1 ± 376.1 ng/g of doxycycline was present in the brains tested (Figure 2.2A). As expected and in accordance with DOXY's pharmacological profile, the levels of DOXY measured in the HI brains increased over the time points examined (Figure 2.2B).

2.3.2. Fluoro-Jade Staining For Degenerating Neurons

In mild HI injury with acute survival times, the numbers of Fluoro-jade positive cells were low compared to animals with a more severe injury (Figure 2.3). Degenerating cells were seen as bright illuminations above background, in discrete patches throughout the right cortex, striatum, thalamus and hippocampus of most vehicle-treated HI animals. As predicted, those pups allowed to live up to 4 hours post-HI had the most Fluoro-jade positive/degenerating cells.

2.3.3. Immunohistochemistry and Western Blotting

Histopathologic evaluation of brain sections reveal that within four hours of HI injury, neuronal cell death processes are initiated and neuronal cell loss is present in the hemisphere ipsilateral to CCA occlusion. Characteristic of a mild injury in an acute survival setting, diffuse and patchy cell loss, as well as neuronal disorganization and loosening of the neuropil were present in the vehicle-treated pups (Figure 2.4).

Cleaved caspase-3 immunoreactivity was seen as coarse, dark, granular deposits within cells of both the ipsilateral and contralateral hemispheres in pups from all treatment groups. Figure 2.5 represents the basal level of cleaved caspase-3 seen in the CA1 region of the hippocampus in SHAM animals (A), as well as the amount of cleaved caspase-3 observed in vehicle treated (B, D, F) and DOXY-treated pups (C, E, G). Increasingly more cleaved caspase-3 positive cells were observed as survival time was increased to 4 hours. Treatment with DOXY significantly attenuated this increased expression. The Western blotting performed was consistent with the immunohistochemical results. It was found that DOXY decreased cleaved caspase-3 protein expression (Figure 2.6) and increased caspase-3 protein expression as compared to vehicle treated HI pups (Figure 2.6A). It was observed that as quickly as one hour after HI in the striatum of a P7 rat, there was a significant upregulation of cleaved caspase-3 protein expression and that the administration of DOXY significantly attenuated this upregulation

As doxycycline is an anti-inflammatory compound, we also investigated activated microglia at the same time points. Figure 2.7 displays representative photomicrographs of ED-1 positive microglia, dorsal to CA1 of the hippocampus, in SHAM (A), vehicle-treated (B, D, F), and DOXY-treated pups (C, E, G). As seen with cleaved caspase-3 expression, basal levels of ED-1 positive microglia were observed in the SHAM pups, and increases in the numbers of activated microglia were observed as quickly as 1-hour post HI. Treatment with DOXY decreased the numbers of activated microglia, especially as time elapsed into the longer survival times investigated.

2.3.4. Amino Acids in the Hippocampus

HI in P7 rat pups significantly increased glutamate levels in the right hippocampus 4 hours post-HI (HI+VEH=15.8±3.1 vs. Control=11.8±1.4 ng/µg; Figure 2.8A) ($p<0.05$). The pups treated with DOXY did have lower glutamate levels (13.1±2.4 ng/µg) when compared to VEH-treated pups, but these values did not reach significance. Hypoxia-ischemia also significantly increased ALA, and GLY levels 2 and 4 hours post-HI, and significantly elevated levels of GLN, SER at most time points examined (Figures 2.8B, D, E, and F). Levels of GABA were significantly increased in VEH and DOXY-treated pups, 30 minutes, 1 and 2 hours post-HI but by 4 hours levels of GABA were no longer significantly different than controls (Figure 8C).

Pups treated with DOXY had significantly lower alanine (HI+VEH=1.12±0.23 vs. HI+DOXY=0.54±0.12 ng/μg), serine (HI+VEH=1.40±0.46 vs. HI+DOXY=0.66±0.12 ng/μg), and glycine (HI+VEH=1.24±0.27 vs. HI+DOXY=0.82±0.17 ng/μg) levels in the hippocampus, 4 hours post-HI (Figure 8B, F, D).

In the hippocampus contralateral to CCA occlusion, levels of ALA increased in response to hypoxia at all time points. DOXY-treated pups had significantly increased GABA and GLN levels as compared to VEH-treated pups, 2 hours post-HI (data not shown).

2.3.5. Amino Acids in the Striatum

In the striatum after HI, glutamate levels increased as compared to control pups although these increases were not statistically significant (Figure 2.9A). In addition, VEH-treated pups had significantly elevated levels of alanine when compared to controls, 30 minutes, 2 and 4-hours post-HI (Figure 2.9B). HI also increased levels of serine in the striatum (Figure 2.9D). As seen in the hippocampus, levels of GABA increased in VEH and DOXY-treated pups, 30 minutes ($p<0.05$), and 2 ($P < 0.05$)

hours post-HI, but by 4 hours levels of GABA were not significantly different than controls (Figure 2.9C).

Administration of DOXY normalized alanine levels 2 and 4-hours post-HI, as DOXY-treated pups had significantly lower alanine levels as compared to vehicle treated HI pups (HI+VEH=1.15±0.47vs. HI+DOXY=0.58±0.13 ng/µg at 4 hours and HI+VEH=2.45±0.65vs. HI+DOXY=1.47±0.90 ng/µg at 2 hours, Figure 2.9B). DOXY-treated pups had significantly lower serine levels as compared to VEH-treated pups, 4-hours post-HI (HI+VEH=1.54±0.36vs. HI+DOXY=0.96±0.15 ng/µg, Figure 2.9D). Treatment with DOXY decreased levels of glutamate, but again these reductions were not significant (Figure 2.9A). HI pups treated with the saline vehicle were observed to have elevated levels of GLN 2 and 4-hours post HI, and treatment with DOXY decreased the levels, but these changes were not significant (data not shown). In addition, treatment with DOXY also decreased levels of ASN, GLY, and TAUR compared to VEH-treated pups although these reductions failed to reach statistical significance (data not shown).

Analyses of the amino acids in the contralateral striatum revealed few distinct trends in the levels of GLU, GABA, ALA, GLN, ASP, GLY, TAUR between DOXY and VEH-treated pups or when compared to control pups at any time point. Four hours post-HI however, DOXY-treated pups had significantly lower levels of serine than VEH-treated pups (data not shown).

2.3.6. Amino Acids in the Frontal-Parietal Cortex

In the ipsilateral frontal cortex after HI injury, glutamate levels from neither VEH-treated nor DOXY-treated pups were significantly different from control (Figure 2.10A). At all time points examined, levels of ALA, SER and GABA in VEH-treated pups were significantly elevated compared to the levels in controls (Figure 2.10B, 2.10C, 2.10F). Thirty minutes and 4 hours post-HI, levels of GLY were significantly elevated beyond the values observed in the control pups (Figure 2.10D). In addition, levels of GLN were significantly elevated 4 hours post-HI (Figure 2.10E).

Treatment with DOXY reduced levels of alanine (HI+VEH=1.14±0.18vs. HI+DOXY=0.59±0.45 ng/μg), GABA (HI+VEH=1.01±0.15vs. HI+DOXY=0.73±0.14 ng/μg), SER (HI+VEH=1.01±0.30vs. HI+DOXY=0.47±0.28 ng/μg), and GLY (HI+VEH=1.77±0.34vs. HI+DOXY=1.06±0.61 ng/μg) in the frontal cortex, 4 hours post HI ($p<0.05$) (Figure 2.10).

In the contralateral, hypoxic hemisphere, those pups treated with DOXY had significantly lower GLU, ALA, ASN, GLN, and aspartate levels when compared to controls, 4 hours-post HI (data not shown). In addition, administration of DOXY significantly reduced levels of GLY 1 hour post-HI and levels of GLN 2 hours post-HI (data not shown).

2.4. Discussion

Previous studies in the literature have shown that excessive excitation, excitotoxicity, and synaptic overload are common events during ischemia or other times of energy failure, when disproportionate amounts of neurotransmitters are released but compensatory rescue mechanisms located in the neuronal membranes are underpowered (Johnston 1995). Independently, amino acid neurotransmitters are closely linked to the vulnerability of the immature brain to neuronal injury (Ikonnmidou *et al.* 2001, Johnston 1995, Johnston & Ishiwa 1995). Glutamate however, has trophic influences in the developing CNS and promotes proliferation and migration of neuronal progenitors and influences synaptic plasticity (Guerrini *et al.* 1995, Ikonnmidou *et al.* 2001, Komuro & Rakic 1993). Data from studies performed in immature rats indicate that these animals may be especially susceptible to elevated concentrations of not only glutamate, but aspartate, glutamine, and GABA as well (Gordon *et al.* 1991, Wallin *et al.* 2000, Cataltepe *et al.* 1996, Puka-Sundvall *et al.* 1997). After HI, glutamate uptake from the synapse is impaired and additional synaptic accumulation of glutamate may result from increased release from neurotransmitter or metabolic pools (Martin *et al.* 1997, Gordon *et al.* 1991). As it is hypothesized that elevated synaptic concentrations of endogenous glutamate may be especially neurotoxic at early developmental stages (Silverstein *et al.* 1991), investigations of these amino acids and others, in addition to therapies that affect them, is important to understanding the evolution of developmental brain damage after HI.

Our data show that in addition to significantly increased microglial activation, cleaved caspase-3 protein expression and neuronal cell loss, levels of glutamate in the hippocampus were significantly increased 4h post-HI. In the striatum and cortex, glutamate levels were increased compared to controls however the values did not reach statistical significance. Similarly, treatment with DOXY variably decreased glutamate, but again not to the degree of statistical significance. It is possible that increased glutamate release from neurotransmitter and neuronal pools and/or suppressed neuronal and glial reuptake could contribute to the elevations observed (Gordon *et al.* 1991). These changes cannot be overlooked, as even minute alterations in neurochemistry may be very important in the developing CNS. Furthermore, the glutamate elevations observed reflect HI injury of a mild nature and it is believed that even small elevations in the immature brain can be neurotoxic, as the balance between glutamate as a neurotrophic factor and glutamate as an excitotoxin is shifted. Moreover, studies in human infants indicate that levels of glutamate, taurine and aspartate may increase according to the severity of hypoxic-ischemic encephalopathy and degree of abnormal outcome observed (Gucuyener *et al.* 1999). DOXY's ability to decrease glutamate may be a direct result of its anti-inflammatory properties. Microglia are known sources of amino acids, and as we have shown, these cells are activated after HI. Therefore, DOXY's ability to inhibit microglial activation after HI may decrease release of glutamate and additional amino acids from these cells.

Like glutamate, GABA has trophic roles during early brain development and as such, interference with GABAergic transmission may affect neuronal wiring, neuronal network plasticity and disrupt neuronal organization (Herlenius & Lagercrantz 2001). It is generally accepted that GABA levels increase after cerebral ischemia to counterbalance increases in glutamate. However, GABA levels do not remain elevated as brain injury evolves. Our data are consistent with this pattern. We found that GABA is significantly increased 30 minutes, and 1 and 2 hours after HI. This may be attributed to increased activity of glutamate decarboxylase (GAD) by acidosis, adenosine triphosphate deficiency, and/or inhibition of GABA transaminase (GABA-T), as this enzyme requires NAD^+ and requires a high pH for optimal functioning (Baxter 1976). We also saw a robust decrease in GABA levels, four hours post-HI in all regions examined. This loss is likely attributed to the loss of GABAergic neurons after HI and/or disequilibrium in the GABA shunt, such as changes in GAD activity as a consequence of primary and secondary energy failure.

Many of our observations are indicative of disequilibrium in the GABA shunt. Besides the changes in GABA mentioned above, significant changes in glutamine levels were documented. In the hippocampus, HI increased glutamine in both treated and untreated pups compared to controls. Although this finding was specific to the hippocampus at all time points, and the frontal cortex 4-hours post-HI, it is inferred that HI causes a disturbance in neuronal-glial glutamate-glutamine homeostasis in favour of increased glutamine synthesis in glia (Wallin *et al.* 2000).

As previously mentioned, glycine levels have been associated with the severity of HI encephalopathy observed in human infants (Roldan *et al.* 1999). The primary pool of glycine that was examined and manipulated in this study was the glycine that acts as a co-agonist with glutamate on N-methyl D-aspartate (NMDA) receptors in the cortex, hippocampus and striatum. The alteration of extracellular glycine in concert with glutamate can dramatically alter NMDA-receptor mediated responses (Herlenius & Lagercrantz 2001). Our data demonstrate that glycine levels were increased after HI compared to controls, in all regions and all time points investigated. Treatment with DOXY robustly reduced glycine levels in the hippocampus and cortex 4 hours post HI. These data suggest that HI may be modifying the cerebral synthesis of certain neurotransmitter amino acids, activating glycine production in particular. Typically, glycine is removed from the synaptic cleft by uptake transporters located on glial cells and on presynaptic terminals of glycinergic nerves. However, the forebrain lacks glycinergic neurotransmission and glycine transporters are found exclusively on glia. Glycine transporter-1 (GLYT-1) transporters are capable of regulating NMDA receptor function by controlling the levels of glycine available to act as a co-agonist (Cooper *et al.* 2003). Our data suggest that HI may directly affect the number or function of these transporters. Further, DOXY may act to inhibit GLYT-1 and drugs that inhibit GLYT-1 may prove to be clinically useful to augment NMDA receptor function and indirectly decrease excitotoxicity (Cooper *et al.* 2003).

Another major finding in this study was that HI considerably increased serine levels. Treatment with DOXY reliably and significantly normalized total serine levels 4-

hours post-injury. Serine homeostasis plays a pivotal role in maintaining the microenviroment required for neuronal development and may be subtlety connected to inflammation. Therefore, DOXY-dependent reductions in this amino acid may be yet another indicator of its ability to decrease neuroinflammation and maintain optimal cellular functioning (DE Koning *et al.* 2003). DOXY could also be normalizing serine's synthetic and/or metabolic pathways or preventing its release from glia (Swanson *et al.* 2004). Serine levels are believed to be highest where there is increased cellular proliferation, and elevations induced by HI could be indicative of increased cell genesis in response to injury (DE Koning *et al.* 2003). Serine is also an immediate precursor of glycine, and may function as an endogenous agonist of the glycine site on NMDA receptors (Wang & Zhu 2004, Cooper *et al.* 2003). Taken together, serine likely has an important role in NMDA-mediated excitotoxicity and optimal NMDA receptor function. Further, it is probable serious dysfunction would occur in the brain when serine biosynthesis, catabolism and serine/glycine metabolism are compromised or altered (DE Koning *et al.* 2003). Thus, DOXY's ability to significantly decrease serine may be beneficial and could represent an indirect effect on excitotoxicity, an effort to maintain cellular homeostasis and subtle augmentation of NMDA receptor function in the examined regions.

Cerebral levels of alanine are intimately related to levels of lactate and thus, cerebral pH. As alanine is formed by the transamination of pyruvate from glycolysis, increased alanine may be indicative of increased glycolysis after HI (Korf & Venema 1985, Gordon *et al.* 1991). Through α-ketogluterate and a pyruvate intermediate, alanine is converted to lactate via lactate dehydrogenase. As the amount of lactate in the brain increases, the pH drops. HI consistently and strongly elevated alanine compared to control, in all regions and time points investigated. Two to 4 hours post-HI, those pups treated with DOXY had significantly lower alanine levels. It is clear that DOXY normalizes alanine levels, and part of its beneficial effects could be related to maintenance of homeostasis by stabilizing lactate levels and consequently pH, and the degree of acidosis in the brain.

In this study, we assessed whether the administration of DOXY would successfully penetrate the blood-brain barrier (BBB), reduce microglial activation and cleaved caspase-3 protein expression, and impact the levels of amino acids after mild HI and acute survival. We showed that a single dose of DOXY administered at the onset of hypoxia 1) significantly penetrates the BBB of immature rats as determined by HPLC extraction; 2) successfully decreases microglial activation and cleaved caspase-3 protein expression; 3) upregulates caspase-3 protein expression and may prevent the cleavage of the pro-form of caspase-3 to the active form; 4) promotes neuronal cell survival up to 4-hours post HI; and 5) consistently lowers the levels of alanine, glycine and serine in the cortex, striatum and hippocampus, 4 hours post-HI. In addition, we demonstrated that HI alone significantly alters levels of glutamate, alanine, GABA, glycine, glutamine and serine in all regions examined. These data indicate that DOXY's ability to acutely protect against a mild HI injury is associated with a reduction in neuroinflammation and pathological apoptosis, thereby increasing neuronal cell survival. In addition to these effects, HI alone and in combination with

DOXY significantly changes amino acid neurochemistry in the immature rat brain. As a fine balance between cell death and cell survival exists in the immature CNS, one must be mindful of treating developmental disorders with drugs that affect amino acids. In critical developmental periods, many synaptic connections are being formed and basal levels of apoptotic cell death are extremely high. Transient interference with the action of certain neurotransmitters at these stages and subtle changes in neurochemsitry may be detrimental and may cause additional apoptotic degeneration. Depending on timing, this may negatively affect large populations of neurons and could give rise to new or secondary neurobehavioral and psychiatric disturbances (Ikonnmidou *et al.* 2001). Thus, results from this investigation indicate that changes in neurotransmitter systems via HI and/or subsequent treatment need to be examined in greater detail to elucidate cellular processes crucial to normal development, whilst being aware of the risk for exchanging one developmental disorder for another.

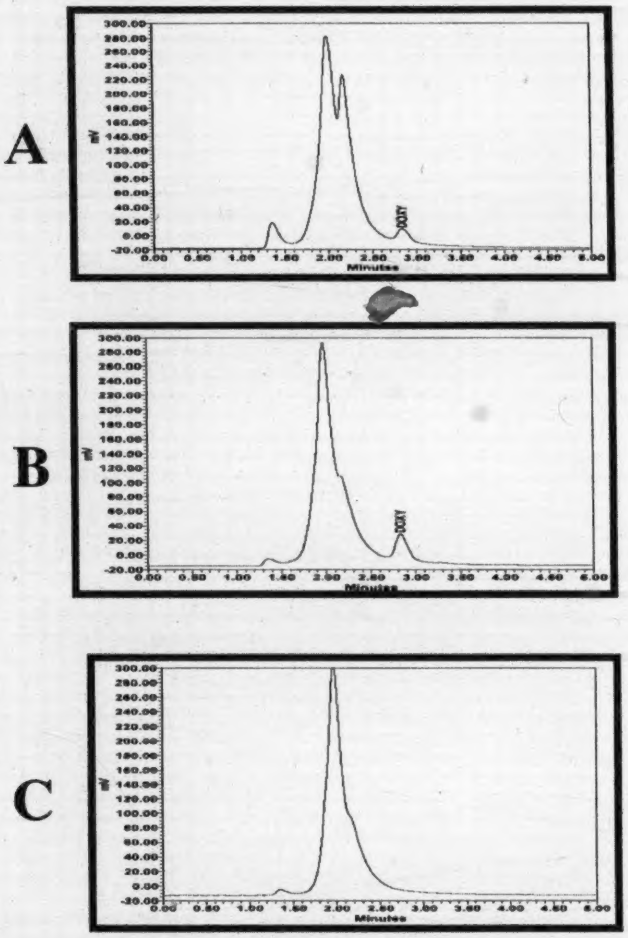

Figure 2.1. Extraction of Doxycycline From the Brains of Hypoxic-Ischemic Rat Pups. Representative chromatograms indicating the characteristic doxycycline peak, seen after HPLC extraction in treated hypoxic-ischemic brains (B), versus the peak of an animal treated with saline vehicle (C). The chromatogram of an extraction performed on the brain of a naïve animal, of which its homogenate was spiked with a known concentration of DOXY (500 ng), is shown in (A). DOXY (10 mg/kg) was administered intraperitoneally as a one-time dose immediately before hypoxia and tissue was collected 4 hours after the HI insult (total time after DOXY administration = 6.5 hours).

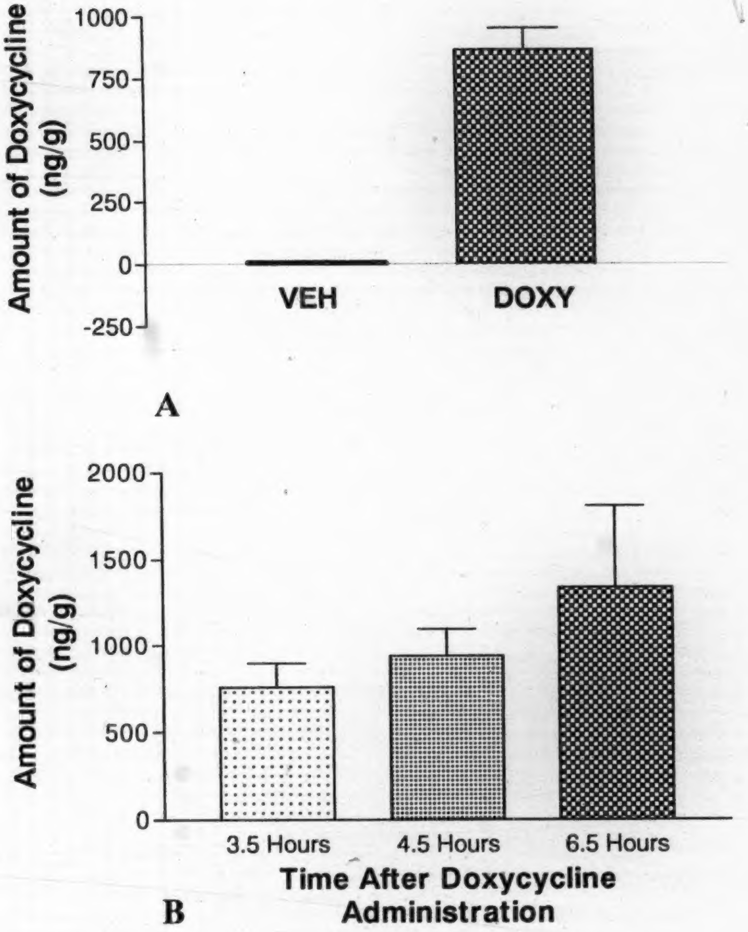

Figure 2.2. Brain Levels of Doxycycline. Results of HPLC extractions (not corrected for instrument or homogenate recovery) indicate that an average level of 867.1 ± 376.1 ng/g of doxycycline was present in the brains enrolled in our study (A). The levels of DOXY present in the HI brains increased over the time points examined (B). DOXY (10 mg/kg) was administered intraperitoneally as a one-time dose immediately before hypoxia and tissue was collected 1, 2, or 4 hours after the HI insult (total time after DOXY administration = 3.5, 4.5 and 6.5 hours).

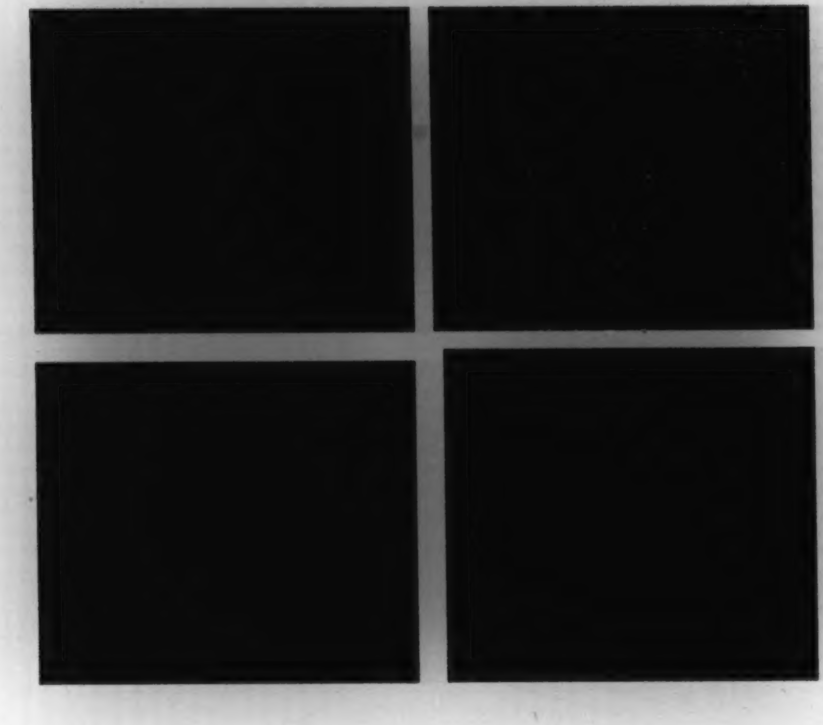

Figure 2.3. Fluoro-Jade Staining For Degenerating Cells. Representative photomicrographs illustrating diffuse and patchy areas of degenerating neurons in the posterior striatum of both DOXY-treated (D) and vehicle-treated pups (C), four hours post-HI. Sham animals have no Fluoro-Jade positive cells (A). The heavily damaged cortex, with immense numbers of degenerating neurons, from a positive control pup (moderate-severe HI) is shown in (B) for comparison. Scale bar = 100μm.

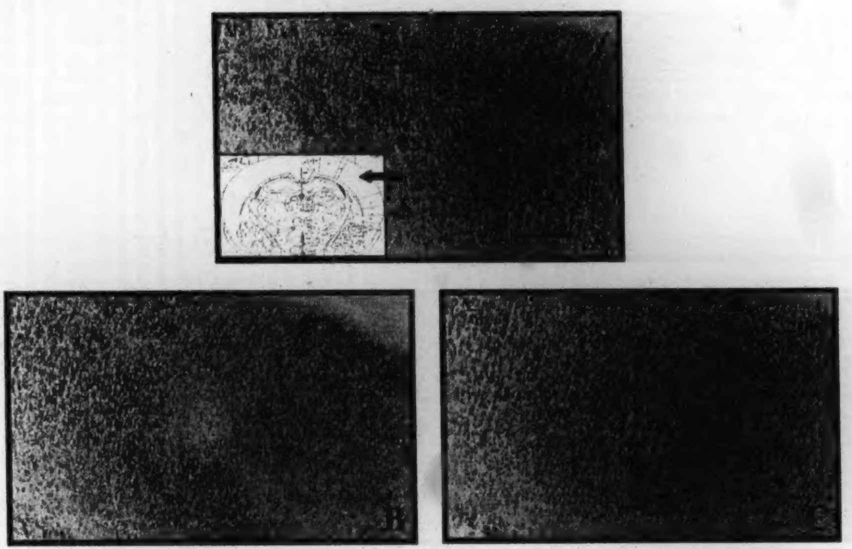

Figure 2.4. Evolution of Neuronal Injury after Hypoxia-Ischemia (HI) in An Acute Survival Setting. Representative photomicrographs show neurons in the frontal-parietal cortex of SHAM pups (A), vehicle-treated (B), and DOXY- treated pups (C), 4-hours post HI. Characteristic of mild HI and acute survival, neuronal changes in the injured pups are limited to decreased expression of NeuN, cortical disorganization, loosening of the neuropil, and patchy cell loss (B). This cell loss was attenuated by the administration of DOXY (C). Inset in (A) represents plate 31 from Paxinos and Watson (1998) and arrow points to area of cortex examined. Scale bar =100μm.

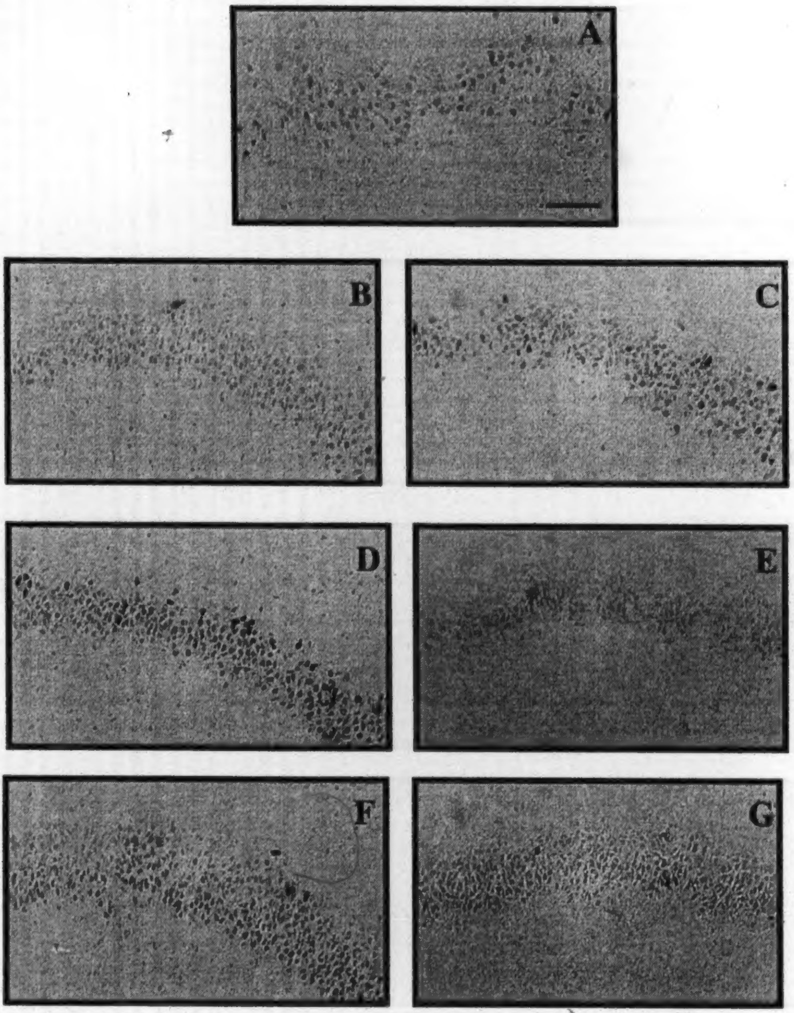

Figure 2.5. Cleaved Caspase-3 Immunoreactivity After Hypoxia-Ischemia (HI) in an Acute Survival Setting. Representative photomicrographs of CA1 hippocampal sections demonstrating that cleaved caspase-3 immunoreactivity is increased in rat pups subjected to HI. Vehicle-treated pups have increased expression of cleaved caspase-3, 2 (D) and 4 (F) hours post-injury. Treatment with DOXY significantly decreased this upregulation at the same time points (Figures E and G, respectively). The basal level of cleaved caspase-3 immunoreactivity as observed in SHAM pups is shown in (A). Plate (B) Vehicle treated pup euthanized 1hour post-HI; Plate (C) DOXY-treated pup euthanized 1 hour post-HI. Scale bar = 50μm.

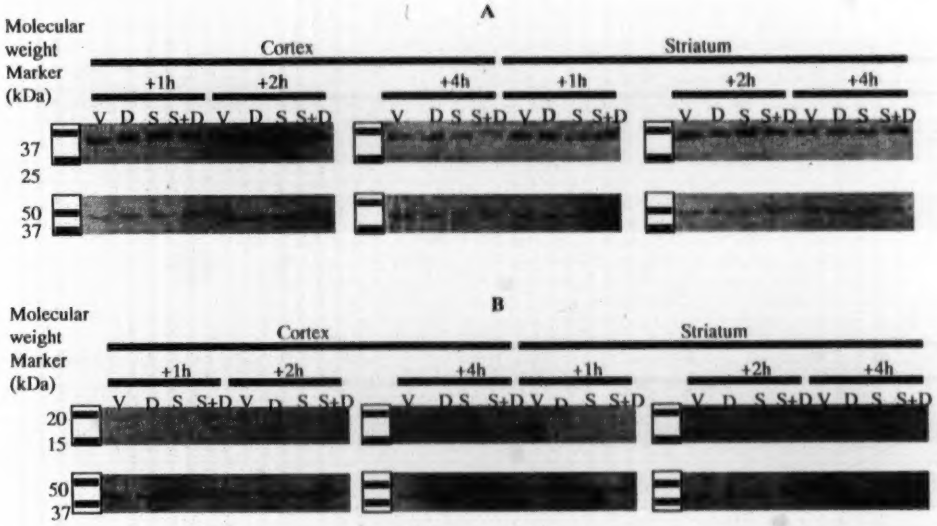
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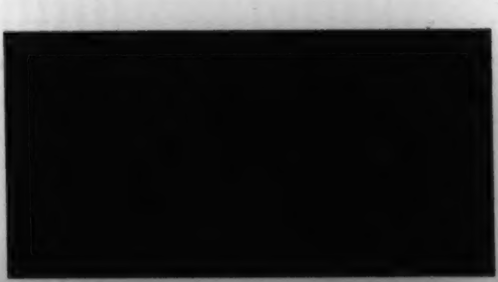
Figure 2.6. Doxycycline Decreases Cleaved Caspase-3 Protein Expression and Increases Caspase-3 Protein Expression After Hypoxia-Ischemia (HI). Representative Western blots depicting decreased cleaved caspase-3 protein expression and increased caspase-3 protein expression in the striatum and fronto-parietal cortex of HI pups in response to treatment with DOXY. Caspase-3 (A) is detected as a single band that runs at approximately 35 kDa, cleaved caspase-3 (B) is detected as a 19-17 kDa doublet. Doxycycline prevents cleavage of and significantly decreases cleaved caspase-3 protein expression as compared to vehicle treated HI pups (B). Non-cleaved caspase-3 builds up in the ipsilateral fronto-parietal cortex with administration of DOXY (A). Blots were reprobed (bottom panels) with an antibody against ß-actin (42kDa) to verify the consistency of protein loading between lanes. (V= vehicle treated; D=DOXY treated; S=SHAM animal; S+D=SHAM animal treated with DOXY)



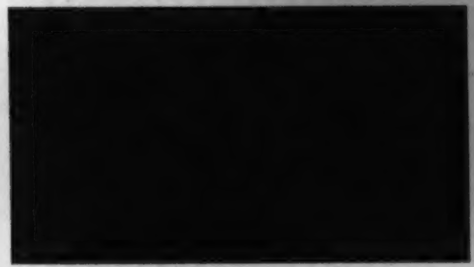


Figure 2.7. ED-1 Immunoreactivity After Hypoxia-Ischemia (HI) in an Acute Survival Setting. Representative photomicrographs showing typical numbers and morphology of ED-1 positive microglia, dorsal to the hippocampus after HI. The basal level of activated microglia observed in the SHAM pups are shown in (A), and increased numbers of ED-1 positive cells were observed as quickly as 1-hour post HI in the vehicle treated pups (B). Treatment with DOXY decreased the numbers of activated microglia, especially as time elapsed into the longer survival times investigated. (Plate C-pup treated with DOXY and euthanized 1 hour post-HI; Plate D-pup treated with vehicle and euthanized 2 hours post-HI; Plate E-pup treated with DOXY and euthanized 2 hours post-HI; Plate F-pup treated with vehicle and euthanized 4 hours post-HI; Plate G- pup treated with DOXY and euthanized 4 hours post-HI). Scale bar = 100μm.

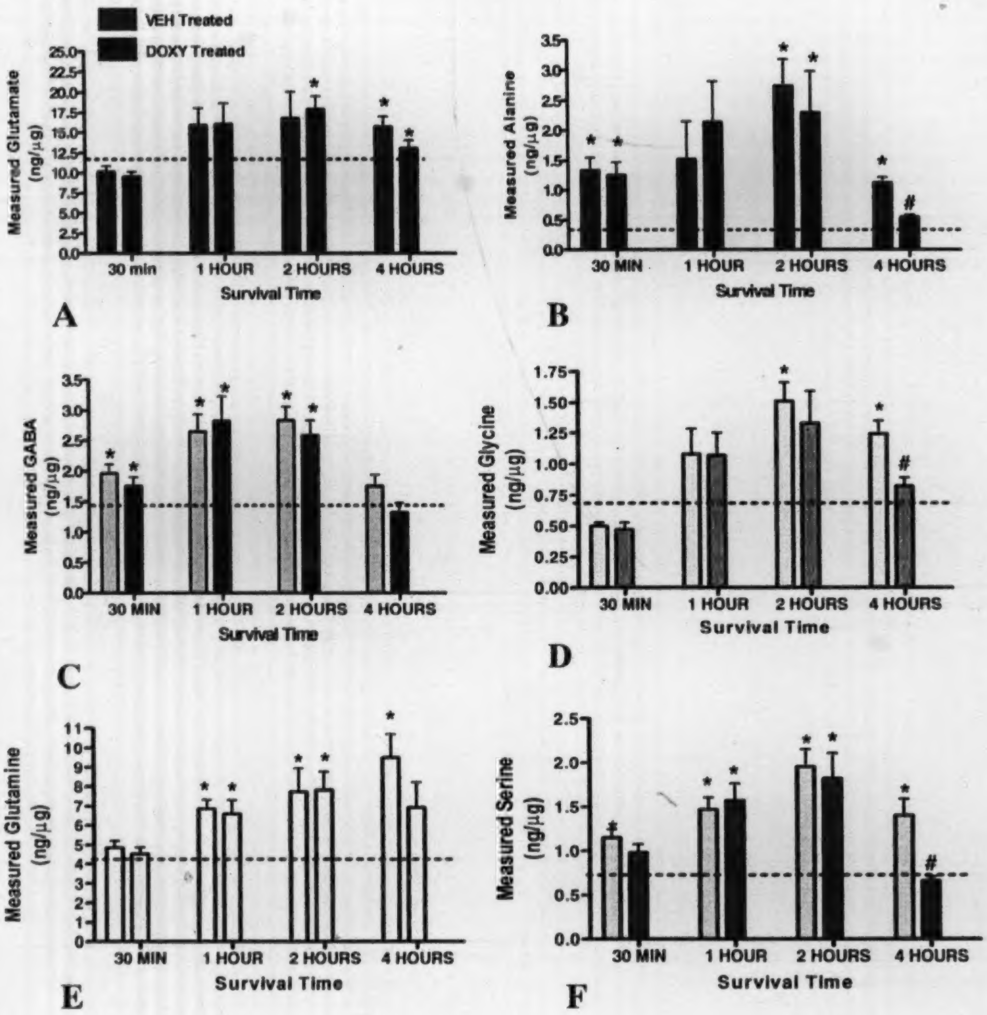


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Figure 2.8. Amino Acid Neurotransmitters in the Hippocampus Ipsilateral to Common Carotid Artery Ligation. Hypoxia-Ischemia (HI) in P7 rat pups leads to significant changes in the concentrations of glutamate (A), alanine (B), GABA (C), glycine (D), glutamine (E), and serine (F) after injury. Hypoxia-ischemia significantly elevated glutamate levels, 4 hours after injury (A). Treatment with doxycycline significantly decreased alanine, serine and glycine, 4 hours post-HI, compared to the levels observed in vehicle treated pups. Values are expressed as ng/μg protein and represent mean ± SD ($n \geq 6$).

Striatum

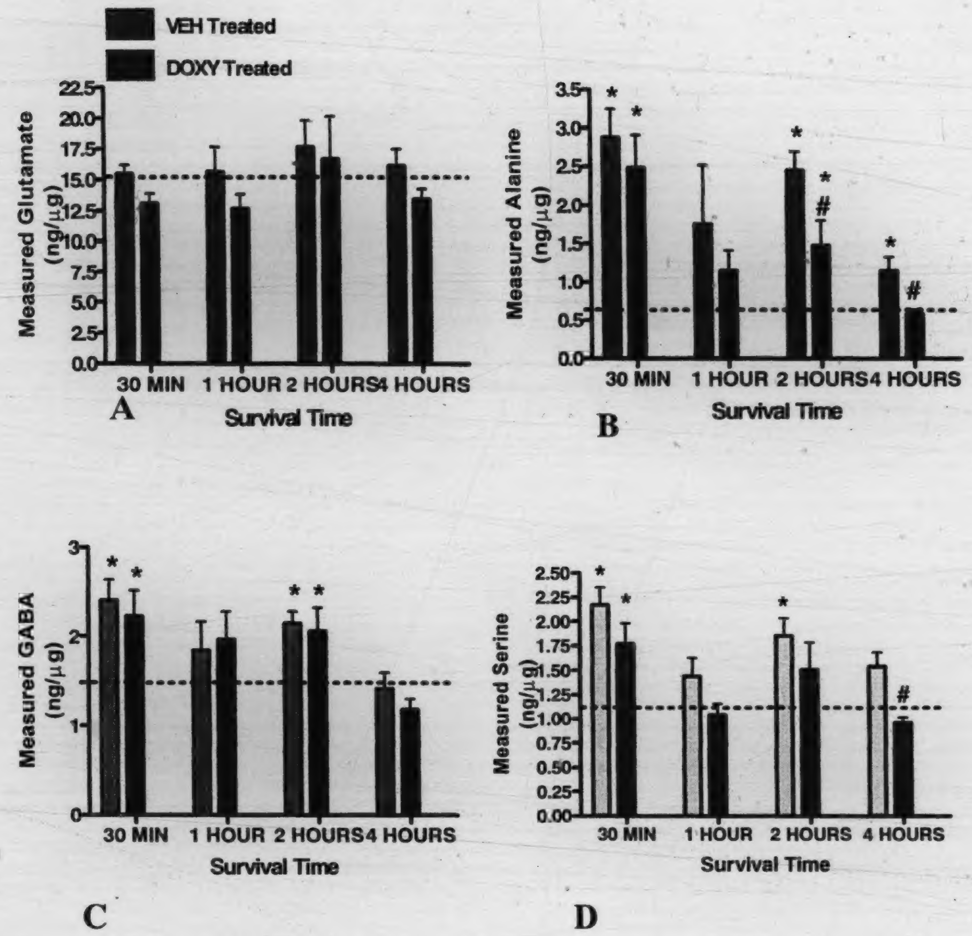

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Figure 2.9. Amino Acid Neurotransmitters in the Striatum Ipsilateral to Common Carotid Artery Ligation. Neonatal hypoxia-ischemia (HI) induced in P7 rat pups results in significant changes to the concentrations of glutamate (A), alanine (B), GABA (C) and serine (D) after injury. When compared to the pups treated with a saline vehicle, administration of doxycycline (DOXY) significantly decreased the levels of alanine, 2 and 4 hours post- HI. In addition, DOXY significantly reduced the levels of serine, 4 hours post-HI as compared to the vehicle treated pups. Values are expressed as ng/μg protein and represent mean ± SD (n≥6).

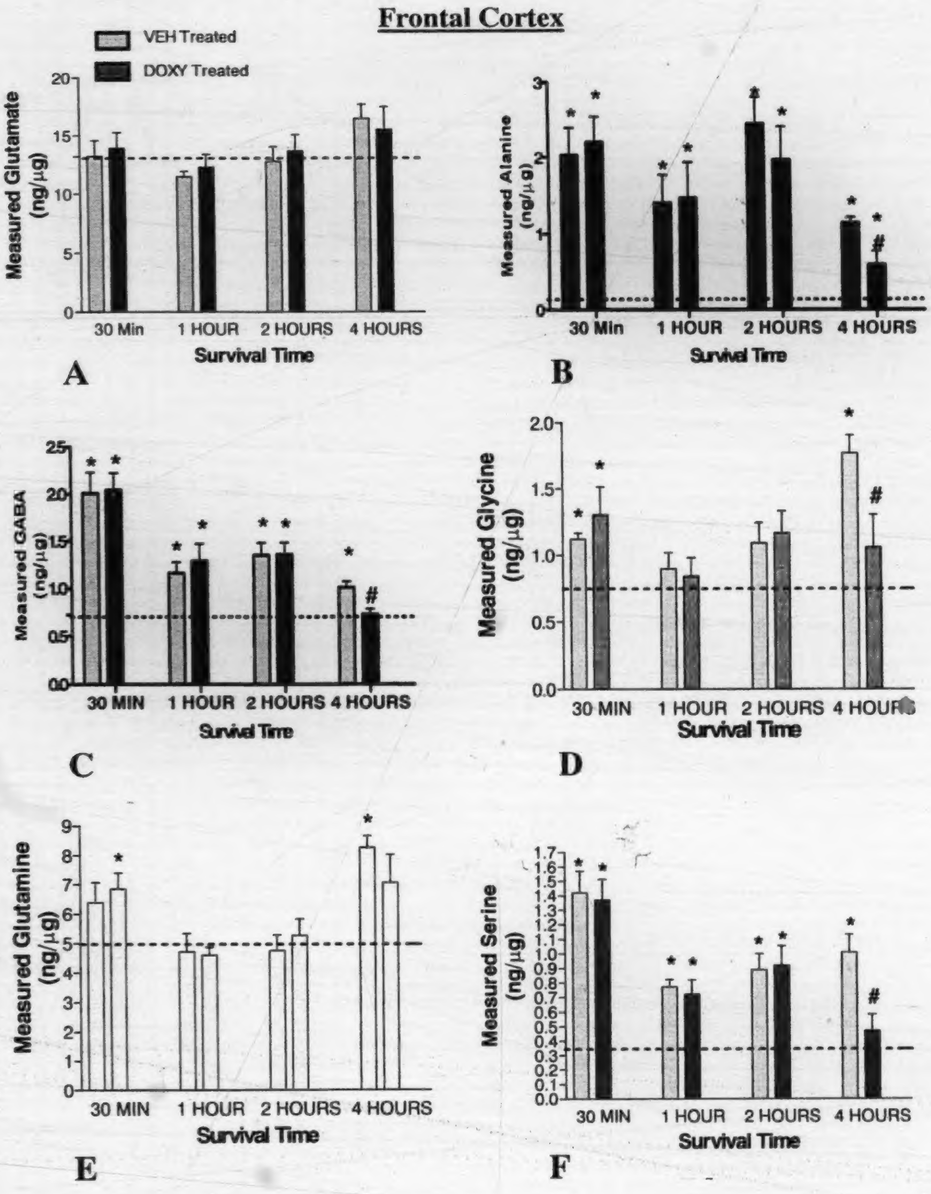

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Figure 2.10. Amino Acid Neurotransmitters in the Frontal-Parietal Cortex Ipsilateral to Common Carotid Artery Ligation. Neonatal hypoxic-ischemic (HI) injury in P7 rat pups results in significant changes in glutamate (A), alanine (B), GABA (C), glycine (D), glutamine (E), and serine (F) after the insult. As compared to the vehicle treated animals, pups treated with doxycycline had significantly lower levels of alanine, GABA, serine and glycine, 4 hours post-HI. Values are expressed as ng/µg protein and represent mean ± SD (n≥6).

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CHAPTER 3

Doxycycline Persistently Reduces Microglial Activation and Cleaved Caspase-3, Whilst Improving Neuronal Survival After Neonatal Hypoxia-Ischemia

* A version of this chapter appears as a published manuscript in the *Journal of Cerebral Blood Flow & Metabolism.* (Jantzie et al., 2005. Doxycycline reduces cleaved caspase-3 and microglial activation in an animal model of neonatal hypoxia-ischemia. *Journal of Cerebral Blood Flow & Metabolism* **25**: 314-324) *

3.1. Introduction

As demonstrated in the previous chapter, hypoxia-ischemia (HI) induces rapid microglial activation and increased caspase-3 expression, leading to acute neuronal cell loss. As well, HI significantly alters the temporal and regional profile of amino acids. Prior to those studies, the acute neuroprotective effects of DOXY in neonatal HI had not been demonstrated and the ability of a tetracycline to affect amino acid neurochemistry had not been reported. Given that we confirmed DOXY's ability to cross the blood-brain barrier and observed a neuroprotective effect over an extremely acute timecourse (up to 4h post-HI), the goal of the investigations presented in this chapter was to expand the survival timecourse and examine DOXY's putative protective effects under neuroprotective and neurorescue dosing regimes with different injury severities.

Hypoxia-ischemia is a common form of perinatal brain damage and is a major contributor to the neurological entities of cerebral palsy, epilepsy, learning disabilities and mental retardation (Vannucci 2000). Hypoxia-ischemia launches a cascade of biochemical alterations that are initiated during the course of the insult and proceed well into the recovery period after resuscitation (Vannucci 1993, Vannucci & Perlman 1997), leading to both immediate and delayed brain cell death. As the window for effective clinical intervention may be particularly narrow in the fetus and the newborn because of the often slow and subtle presentation of the onset of the insult, recent focus has been on defining and blocking the mechanisms underlying delayed or secondary brain injury (du Plessis & Volpe 2002, Nakajima *et al.* 2000).

Key players in delayed brain injury such as apoptosis and the inflammatory response have been reported to play a significant role in the evolution and propagation of the brain injury associated with HI (Arvin *et al.* 2002, Cheng *et al.* 1998, Nakajima *et al.* 2000, Northington *et al.* 2001). Previously, inflammation has been shown to be involved in the pathogenesis of traumatic brain injury, adult cerebral ischemia and neurodegenerative disorders including Alzheimer's and Parkinson's diseases (McGeer & McGeer 1995, Merrill 1992, Yrjanheikki *et al.* 1999). As microglia are the innate inflammatory and resident tissue macrophages of the central nervous system (CNS) (Hanisch 2002, Liu & Hong 2003, Magnus *et al.* 2002, McGeer & McGeer 1995, Streit 2002) and caspase-3 is the main death effector involved in apoptosis (Han *et al.* 2002), the changes in these variables with hypoxia and ischemia in neonatal rats were investigated in the present study.

As described in Chapter 2, Doxycycline (DOXY) is a lipophillic, second-generation tetracycline derivative that has anti-inflammatory actions independent of its anti-microbial actions (Tikka *et al.* 2001, Yrjanheikki *et al.* 1998). It crosses the blood-brain, blood-cerebral spinal fluid and placental barriers and thus has excellent brain and body tissue penetration (Jantzie *et al.* 2006). DOXY and another tetracycline, minocycline (MINO), were previously reported to be neuroprotective in global and focal brain ischemia in adult animals as well as in cell culture models of cell death (Tikka *et al.* 2001, Yrjanheikki *et al.* 1998, Yrjanheikki *et al.* 1999). These drugs significantly protected hippocampal neurons from global and focal ischemia (Yrjanheikki *et al.* 1998) and delayed mortality associated with other types of brain injury such as Huntington's disease (Chen *et al.* 2000). MINO has well documented mechanisms of action; it is known to inhibit the induction of interleukin 1-β converting enzyme (ICE), prevent inducible nitric oxide synthase (iNOS) protein expression, inhibit caspase production and prevent the activation of microglia (Chen *et al.* 2000, Tikka & Koistinaho 2001, Yrjanheikki *et al.* 1998, Yrjanheikki *et al.* 1999). As well, MINO has recently been shown to be neuroprotective in the same animal model of HI as used in our study, even when given hours after the HI insult (Arvin *et al.* 2002). However, MINO has a significant side-effect profile. It has potential for vestibular toxicity (Klein & Cunha 1995) and, through its propensity to bind calcium, markedly affects bone and teeth development. It is therefore not approved clinically for use in neonates. In contrast, DOXY has fewer adverse effects and is approved for use in neonates. Thus, the aim of the investigation presented in this chapter was to investigate the robustness of DOXY's putative neuroprotective abilitity by examining if DOXY, through inhibition of brain microglial cell activation and reduction in caspase-3 activation, would protect the neonatal brain from HI-induced brain injury 7d after both mild and moderate HI.

3.2. Materials and Methods

3.2.1. Animals and Surgical Procedures

As previously described in Chapter 2, all experiments were performed in accordance with the guidelines set forth by the Canadian Council on Animal Care and were approved by the Health Sciences Animal Policy and Welfare Committee of University of Alberta. On post-natal day 7 (P7), Sprague Dawley rat pups were anaesthetized with a mixture of halothane (5% induction, 1.5% maintenance) and oxygen balanced with nitrogen. We used the well-characterized modified-Levine procedure (Nakajima *et al.* 2000, Levine 1960, Rice *et al.* 1981, Vannucci *et al.* 1999) in which unilateral common carotid artery (CCA) ligation is followed by exposure to hypoxia. After CCA ligation, the pups were placed in a humidified hypoxia chamber (premixed 8% O_2 balanced with N_2) for either 1h (mild HI) or 1.5h (moderate HI). Normothermia was maintained in the chamber at 37.5 °C.

3.2.2. Drug Administration and Tissue Preparation

DOXY (SIGMA, 10 mg/kg) or saline vehicle (VEH) was administered intraperitoneally in a volume of 100 μl as a one-time dose immediately before HI, 1, 2, or 3h post HI (n=6). Injections were performed using a 3/10cc diabetic syringe with a 29-gauge needle (Kendal). Pups were euthanized by decapitation seven days after the HI insult and their brains removed and flash frozen in isopentane on dry ice and stored at –80 °C until analysis. For immunohistochemical analyses, serial coronal sections (20 μm) throughout the brain were obtained using a cryostat.

3.2.3. Immunohistochemistry

Immunostaining was performed using antibodies recognizing neurons (mouse anti-neuronal nuclei [NeuN], Chemicon, 1:1000), an apoptotic effector (rabbit anti-human cleaved caspase-3, Cell Signaling Technology, 1:1000), a microglial marker (mouse anti-rat ED-1, Serotec, 1:100), an oligodendrocyte marker (mouse anti-CNPase, Chemicon, 1:250) and an astrocyte marker (rabbit anti-cow glial fibrillary acidic protein [GFAP], Dako, 1:1000). For each animal, immediately adjacent sections were used for each of the primary antibodies. Briefly, fresh frozen sections were post-fixed in buffered formalin and then defatted and dehydrated in a graded series of ethanol washes. Sections were then incubated in a humidifying chamber with 1% hydrogen peroxide to quench endogenous peroxidase enzyme activity and were subsequently blocked with Universal Blocking Serum (Dako) or Normal Horse Serum mixed with Triton-X 100 (0.2% for GFAP, CNPase, NeuN and Caspase-3, and 0.5% for ED-1) to reduce non-specific background staining. Sections were allowed to incubate with the primary antibodies for 1 hour (GFAP, NeuN) or overnight (caspase-3, CNPase and ED-1). After incubation with the species appropriate secondary IgG antibody (all Dako, 1:200) for 30 minutes, the sections were carefully washed and incubated for 30 minutes with an avidin-biotin complex (ABC 1:100, Vector Laboratories), washed 3 times and immunoreactivity visualized with 3,3′ diaminobenzedine tetrahydrochloride [(DAB) Sigma]. In addition to the standard negative control (no primary antibody applied during the course of the immunohistochemistry procedure), a cleaved caspase-3 blocking peptide (Cell Signaling Technology) was also utilized to further evaluate the specificity of the cleaved caspase-3 antibody reactivity. Specifically, 10 μl of cleaved caspase-3 antibody and 10 μl of blocking peptide in 480 μl Tris buffer was incubated at 4°C for 2h before allowing the solution to react to the sections.

The numbers of immunopositive cells in a minimum of 5 fields (depending upon the size of the brain region) in each of the hippocampus, thalamus and cerebral cortex were determined using brightfield microscopy and an unbiased stereological procedure by an investigator blind to the treatment groups. The values obtained in each field were averaged and the means were used in statistical analyses.

As the values obtained were ordinal and normally distributed, and given there were no significant differences in the group variances, parametric statistical analyses (analysis of variance [ANOVA]) were applied to the data to evaluate main treatment effects. Where significant main effects were identified, Newman Keuls multiple comparison tests were run. A probability of $p<0.05$ was chosen to represent statistical significance. Statistical analyses revealed no significant differences in the brains of the obligate control animals at any time point. Therefore, these animals were pooled into a single control group.

Fluorescent immunostaining was performed to corroborate the immunoreactivity seen using the DAB chromagen. The above procedure was used until the application of the secondary antibody. On representative sections, after the excess primary antibody was washed off, an appropriate secondary IgG antibody conjugated with fluorescence was applied for 30 minutes. For the cleaved caspase-3 primary antibody, a donkey anti-rabbit Cy2 conjugate or donkey anti-rabbit Texas Red was chosen. For the NeuN primary, a donkey anti-mouse conjugated to FITC was used, and for the ED-1 primary antibody, an anti-mouse IgG conjugated to Texas Red was applied (1:100 for all). The sections were then rinsed and cover slipped using an aqueous mounting medium to prevent auto-fluorescence.

3.2.3. Fluoro-Jade Staining for Degenerating Cells

Fluoro-Jade staining was performed as previously described in (Jantzie *et al.* 2006). Briefly, sections were fixed in formalin, washed with water, and dehydrated in a series of ethanol washes. Sections were then incubated in a 0.06% potassium permanganate solution for 15 minutes to suppress background staining. Sections were then washed with ddH$_2$0 and were incubated in a 0.001% Fluoro-jade solution made in 0.1% acetic acid, in the dark for 30 minutes. After washing with ddH$_2$O again, the sections were placed in a drying oven held at 37.5 °C until completely dry (30-45 minutes). Sections were then placed in xylene (3x 1 min), and coverslipped with DPX (Fluka).

3.3. Results

No observable alterations in weight gain, suckling or reflexive functions were observed in any pup subject to a mild HI injury during the one-week observational period. The mortality of those pups subjected to mild HI was 0%, whereas the mortality of those pups in the moderate HI group was 8.3%. Mortality after moderate HI was attributed to failure-to-thrive, as these pups were unable to suckle and/or were victims of maternal neglect. On average, those pups with the most severe HI lesions weighed less than the SHAM animals and the pups in the mild HI group.

3.3.1. Neuronal Cell Loss and Degeneration

Histopathological evaluation of the brain sections revealed that the neuronal cell loss observed in the VEH-treated HI animals was diffuse, patchy and present throughout most of the brain, although more severe damage was observed in the hemisphere ipsilateral to the ligated carotid artery (Figure 3.1). Specific areas of the developing brain found to be especially susceptible to this combination of hypoxia and ischemia, as evidenced by significant patterns of neuronal loss, gliosis and numerous Fluoro-jade positive cells, were identified in the dorsal-lateral cortex, striatum, hippocampus, thalamus, and subcortical and periventricular white matter tracts (Figure 3.1). Figure 3.2 illustrates the characteristic pattern of neuronal loss observed in CA1 hippocampus of the hemisphere ipsilateral to carotid ligation after a mild HI injury. In VEH-treated pups, the average loss was consistently observed to be 30% of total cells in this region. Treatment with DOXY at all time points significantly reduced the neuronal loss observed in CA1 to 10% of total cells in this region (Figure 3.2). As clearly evidenced by Figures 3.3, 3.4 and 3.5, significantly more neuronal cell loss and neuronal degeneration was observed throughout the brains of those pups subject to a moderate HI injury. An additional 30 min of hypoxia (90min vs. 60min) resulted in a continuum ranging from the diffuse pattern of injury described above, to infarction, cystic-like lesions, hippocampal compression, severe ventriculomegaly and complete ablation of certain regions (i.e. the dentate gyrus) (Figure 3.3 and 3.4). Moderate HI also significantly increased the number of Fluoro-jade positive cells in all regions examined compared with mild HI (Figure 3.5, hippocampus/cortex). Treatment with DOXY significantly improved neuronal survival in the hippocampus and thalamus of the animals subject to moderate HI (data not shown), especially when administered as a pre-treatment (neuroprotective) or when administered 1h post-HI (neurorescue). However, the degree of neuroprotection or neurorescue was neither as robust nor as consistent as observed in the mild injury.

3.3.2. Cleaved Caspase-3 Immunoreactivity

Cleaved caspase-3 immunoreactivity was seen as dense, dark and course granular deposits within cells. Figure 3.6 illustrates the specificity of the cleaved caspase-3 immunostaining performed and shows the difference in staining patterns in sections exposed to the cleaved caspase-3 antibody and a specific blocking peptide, as well as the difference in the caspase-3 immunoreactivity in the ipsilateral and contralateral cerebral cortex. It was found that cleaved caspase-3 immunoreactivity was increased in the ipsilateral but not contralateral side of the HI brains. Using antibodies specific for neurons (NeuN) and cleaved caspase-3, and appropriate secondary antibodies conjugated to fluorochromes, double staining was performed to pinpoint the cellular location of the activated caspase-3. Activated (cleaved) caspase-3 was observed co-localized to neurons (Figure 3.7A-C). Large numbers of cleaved caspase-3 positive non-neuronal cells were also seen in white matter, and these were identified as oligodendrocytes (Figure 3.7D). Figure 3.8A displays representative photomicrographs of caspase-3 immunoreactivity in the hippocampus of brain sections obtained from SHAM, VEH-treated and DOXY-treated rat pups subject to mild HI. There was a basal level of cleaved caspase-3 immunoreactivity observed in the SHAM animals. Figure 3.8B displays quantitative analyses of cleaved caspase-3 immunoreactivity in the mild HI animals. The number of cells positive for cleaved caspase-3 was significantly increased ($p < 0.05$) in all VEH-treated HI animals (48±3.0) compared to the SHAM (16±2.0) and all DOXY-treated animals (Pre DOXY+ HI=16.7±2.8; DOXY 1 hour post HI = 18±1; DOXY 2 hours post HI = 21±1.3; DOXY 3 hours post HI = 30±2.5). Further, the numbers of cleaved caspase-3 immunopositive cells were increased in the animals that received DOXY 3 hours post HI compared to animals that received DOXY pre-HI, 1 and 2 hours post HI, although this was not significant. Similar results were observed in the thalamus and cerebral cortex (data not shown). The basal levels of caspase-3 activation were quantified in the SHAM-operated animals and it was found that DOXY did not affect basal caspase-3 activation. Fluorescent immunostaining corroborated the decrease in the caspase-3 immunoreactivity with the administration of DOXY (data not shown). These patterns and trends were replicated in the animals subject to a moderate HI, however significantly more cleaved caspase-3 was seen in all regions compared to the mild animals (data not shown).

3.3.3 Microglial Activation

As we had already documented DOXY's acute anti-inflammatory action, we evaluated the ability of DOXY to persistently inhibit microglial activation in this investigation. Figure 3.9 and 3.10 displays representative photomicrographs of the hippocampus in brain sections obtained from SHAM, VEH-treated and DOXY-treated HI rat pups 7d post-HI. In the SHAM animals few activated microglia were observed. ED-1 positive microglial cells in this group maintained the ramified morphology of resting microglia (Figure 3.9A plate(a)). In contrast, in sections obtained from the VEH-treated HI pups, the microglia displayed dramatic morphological changes from resting ramified cells to activated spheroid microglia and became more numerous in response to injury (Figure 3.9A(b)). After moderate HI, significantly more of these activated, spheroid microglia were observed in all areas of the brain as compared to the microglia located in discrete patches of the corpus callosum, hippocampus, dorsal lateral cortex and thalamus of those brains subject to mild-HI. Figure 3.9 displays representative photomicrographs and quantitative analyses of ED-1 immunopositive cells in mild HI. ED-1 immunoreactivity was significantly increased ($p < 0.05$) in VEH-treated HI (24 ± 2.6) compared to SHAM (10±0.70) and DOXY treated animals (Pre DOXY + HI = 7±0.4; DOXY 1 hour post HI = 12±3.0; DOXY 2 hours post HI = 13±1.80; DOXY 3 hours post HI = 16±2.4). It was found that the inhibition of microglial activation occurred in a time-dependent manner. Further, as shown in Figure 3.9, the numbers of ED-1 immunopositive cells were increased in animals that received DOXY 3 hours post-HI compared to animals that received DOXY pre-HI, 1 and 2 hours post-HI, although this was found not to be statistically significant. Again, similar results were observed in the thalamus and cerebral cortex (data not shown). Fluorescent staining corroborated the reduction in activated microglia with DOXY administration (data not shown). Figure 3.10 shows the typical pattern and distribution of activated microglia in the thalamus of moderate HI brains, and a time-dependent response to DOXY administration.

3.3.4 Reactive Astrocytosis

Reactive astrocytes were observed in all animals in the study. GFAP was heavily expressed in mature astrocyte processes and its expression increased after injury with concomitant changes in the morphology of astrocytes. The basal level of reactive astrocytes present in a SHAM animal is shown in Figure 3.11A. These astrocytes were very delicate, with small cell bodies and thin projections. In contrast, the astrocytes observed in the vehicle-treated mild HI pups (Figure 3.11B) were hypertrophic, with large, swollen cell bodies and thick projections. With administration of DOXY, astrocytes were less hypertrophic, less intensely GFAP immunoreactive, and exhibited thinner projections, similar to those observed in the SHAM animal. Consistent with our reports on caspase-3 and ED-1 immunoreactivity, the GFAP immunoreactivity and astrocyte morphology changed in a time-dependent fashion with administration of DOXY after the HI insult.

3.4. Discussion

In this study we assessed whether DOXY would protect the neonatal rat brain from HI-induced brain damage in a sub-acute survival setting extending to 7d post-injury. We demonstrated that a single dose of doxycycline administered immediately before, 1, 2, or 3 hours post-HI to neonatal rats significantly decreased apoptotic signaling (caspase-3 immunoreactivity/protein expression), microglial activation (ED-1 immunoreactivity) and promoted neuronal cell survival. Further, we demonstrated the differences in pattern and expression of cleaved caspase-3 and activated microglia between mild and moderate HI brain injury and further elucidated the time-dependent effects of DOXY administration in different severities of HI.

Previous studies have shown that a significant proportion of neuronal cell death in brain development and pathology has features of apoptosis, with prominent caspase-3 activation (Gill *et al.* 2002, Pulera *et al.* 1998, Roth & D'Sa 2001, Silverstein *et al.*

1997, Cheng *et al.* 1998, Morrison *et al.* 1998, Nakajima *et al.* 2000, Northington *et al.* 2001). Cleaved caspase-3 immunoreactivity was found in both hemispheres of the neonatal brain subjected to HI. It was found that the ipsilateral side of the HI brain had more caspase-3 immunoreactivity and more neuronal loss as compared to the contralateral side, indicating the consistency in the chosen model and the expected pattern of brain damage with this preparation (Cheng *et al.* 1998, Vannucci *et al.* 1999). With respect to our results, DOXY's mechanism of action was associated with a significant decrease in the activation of caspase-3 and thus, a reduction in the amount of apoptosis that occurred during HI injury. Co-localization of cleaved caspase-3 with fluorescent NeuN and CNPase indicated that both neurons and oligodendrocytes were dying via apoptosis and thus, DOXY was attenuating the production of this final effector of apoptotic cell death in both gray and white matter. The decrease in apoptotic cell death was observed in various vulnerable regions, after both mild and moderate HI, throughout the neonatal brain including the CA1 region of the hippocampus as well as, the dentate gyrus, cortex and striatum (data not shown). The decrease occurred in a DOXY-administration, time dependent manner, and strongly suggests that DOXY may promote neuronal survival by interfering with pathological apoptosis and consistent with the reports in Chapter 2, preventing the cleavage of the pro-form of caspase-3 to the active form.

Our results are consistent with the previous notion that apoptosis may be the most prominent cell death mechanism in the neonatal brain as compared to adult animals (Gill *et al.* 2002, Hu *et al.* 2000, Pulera *et al.* 1998). It is thought that the involvement of caspase-3 in the pathogenesis of cell death after HI declines during neuronal maturation and that caspase-3 plays a major role in the death of immature neurons after acute HI (Hu *et al.* 2000, Wang *et al.* 2001). Other studies have shown that inhibition of caspases protects neurons against HI-induced injury and protects the neonatal rat brain from HI injury (Cheng *et al.* 1998, Han *et al.* 2002). It is therefore postulated that inhibition of caspase-3 is an important aspect of neuroprotection associated with neonatal HI. Thus, the ability of DOXY to promote cell survival via the decrease in caspase-3 expression provides a mechanism of action for DOXY and illustrates its potential as a therapy for HI brain injury. In addition, we show that increasing the severity of HI results in increased distribution and many more Fluoro-jade positive cells. These data indicate that the proportion of cell death attributable to apoptosis post-HI may be severity-dependent, as although the exact mechanism of Fluoro-jade staining is not known, it is thought to label cells dying independent of caspase machinery.

Our data also show that DOXY significantly reduced ED-1 immunoreactivity, a marker for activated microglia/macrophages, confirming that DOXY is a potent inhibitor of microglia. As cells of macrophage potential, microglia need appropriate stimulation to enter a stepwise transformation to develop the features and functions of full-blown macrophages (Hanisch 2002). Blocking the activation of microglia may inhibit phagocytosis, inhibit release of cytotoxic molecules, preclude entry into certain cell death pathways, and thus prevent the subsequent demise of neurons that occurs after the activation of these cells.

GFAP immunohistochemistry was undertaken in these investigations because damage to the CNS is usually accompanied by hypertrophy and hyperplasia of astrocytes; therefore, increased GFAP immunoreactivity was taken as a reliable marker of CNS insult (Todd & Butterworth 2001). In addition to its role as an inhibitor of apoptosis and microglial activation, DOXY had modulatory effects on astrocytes. DOXY did not reduce the number of reactive astrocytes but produced an altered morphology of the astrocytes present. Our results corroborate previous data that suggests that the mechanisms of action associated with the activation of microglia and astroglia are independent of each other and mediated through different pathways (Yrjanheikki *et al.* 1998), in that the administration of DOXY did not affect the number of astrocytes present after HI but did reduce the number of activated microglia present after HI. It also furthers the notion that activated microglia but not necessarily astrogliosis, contributes to ischemic neuronal cell death and provides more evidence that astrocytes are fulfilling neuronal support roles in HI (Yrjanheikki *et al.* 1998).

Taken together, the findings from this study indicate that systemic administration of doxycycline protects the neonatal from HI in a sub-acute survival setting. Especially in the clinically relevant mild HI model, we found that even when administered 3 hours after the insult, DOXY reduced microglial activation, decreased cleaved caspase-3 protein expression and significantly improved neuronal survival. Doxycycline may prove to be an attractive pharmacological therapy because of its ability to protect the brain against pathological apoptotic neuronal cell death and neuroinflammation secondary to brain injury. Furthermore, DOXY has the least toxic side effects of the tetracyclines and is already approved for the use in neonates as an antibiotic. Thus, the results gained from this study indicate that doxycycline may have therapeutic potential in the prevention of neuronal cell death due to perinatal/neonatal hypoxia-ischemia.

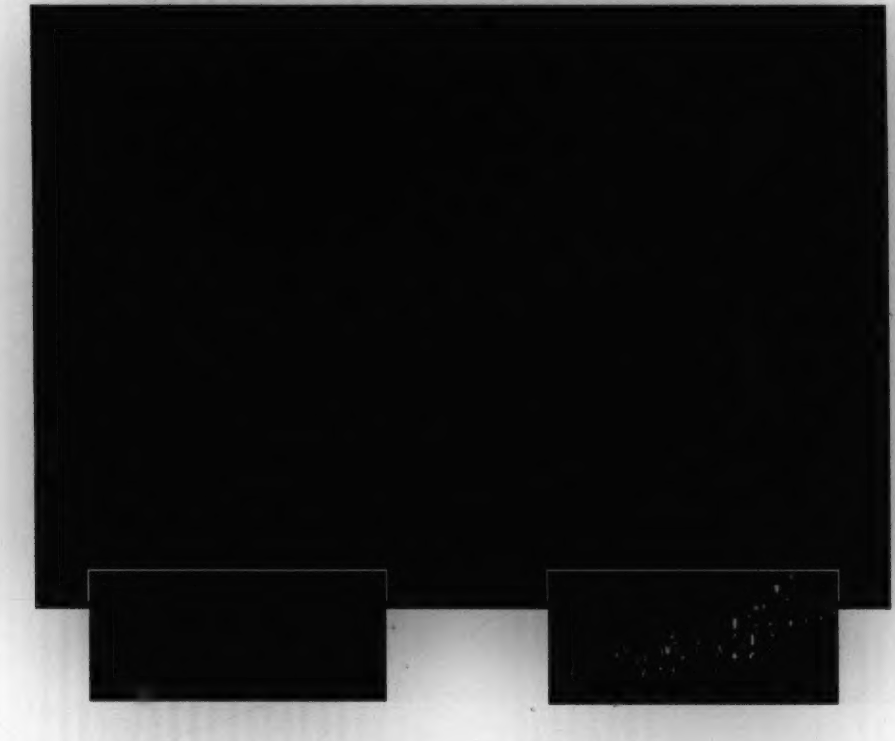


Figure 3.1. Fluoro-Jade Staining For Degenerating Cells in the Hemispheres Ipsilateral and Contralateral to Common Carotid Artery Ligation. A moderate hypoxic-ischemic brain injury results in numerous degenerating cells throughout the hippocampal formation and many nuclei in the thalamus (B). The unilateral nature of this injury is clearly depicted as there is an absence of Fluoro-jade positive cells in the hemisphere contralateral to carotid artery ligation (A). Scale bars = 100μm and 50μm inset.

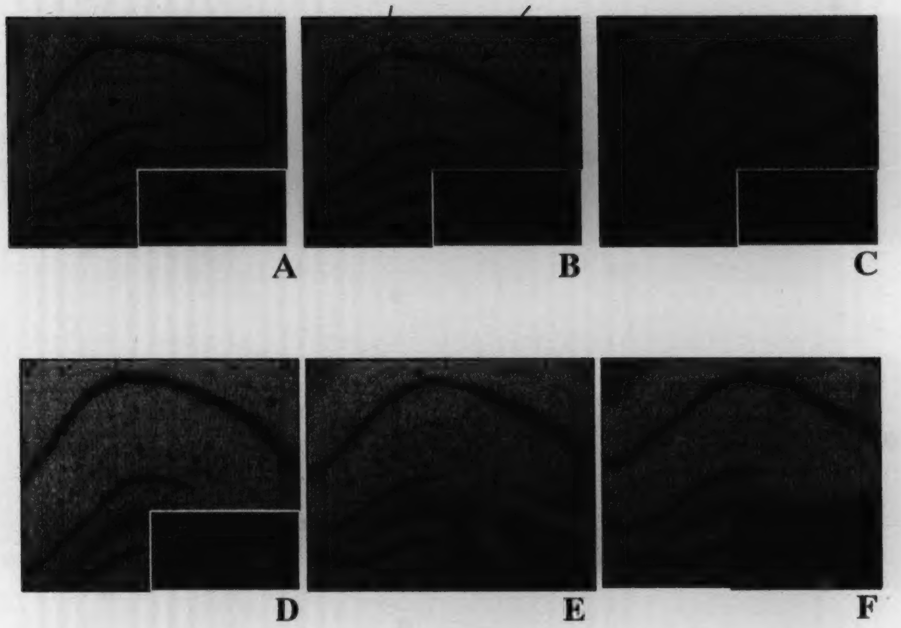


Figure 3.2. Neuronal Cell Loss in the Hippocampus After Mild Hypoxia-Ischemia. Doxycycline (DOXY) attenuates neuronal loss in the hemisphere ipsilateral to carotid ligation in hypoxic-ischemic rat pups. Representative photomicrographs illustrating patchy neuronal cell loss in the hippocampus of vehicle-treated HI rat pups (B) is attenuated by administration of DOXY pre HI (C), 1 hour post HI (D), 2 hours post HI (E) and 3 hours post HI (F). SHAM animal is shown in (A). Scale bars = 100μm and 20μm inset.

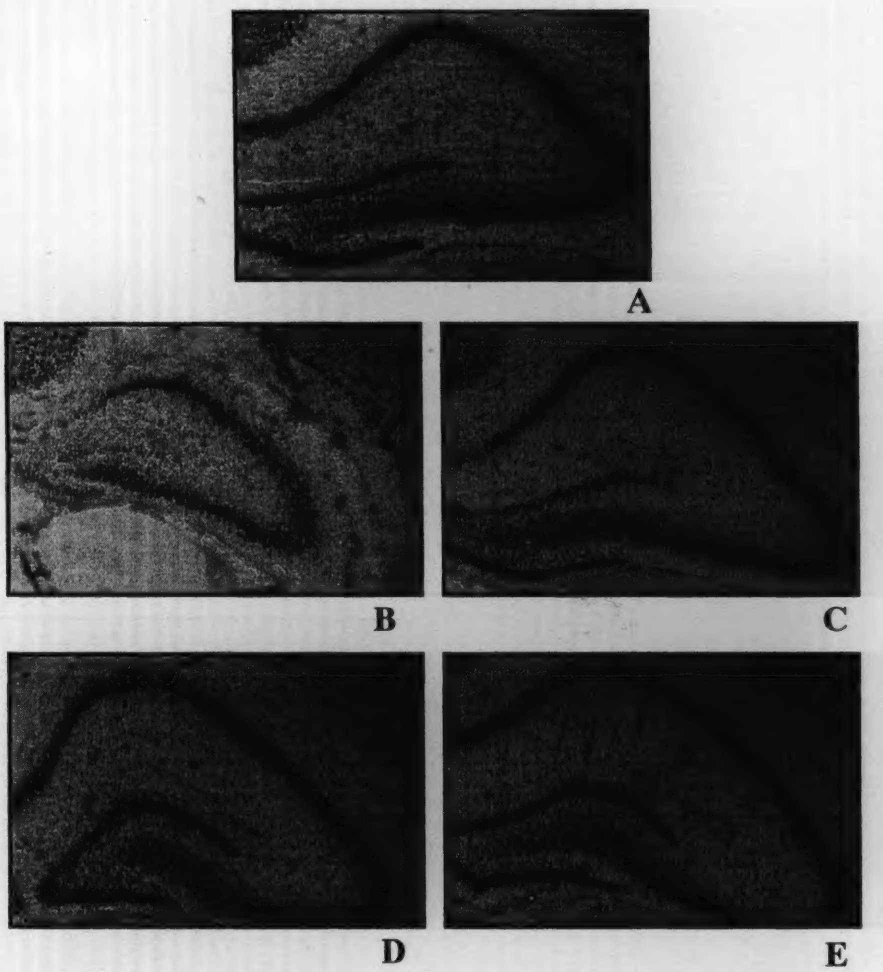


Figure 3.3. Neuronal Cell Loss in the Hippocampus After Moderate Hypoxia-Ischemia. Representative photomicrographs illustrating hippocampal compression, and ventriculomegaly in vehicle-treated HI rat pups (B) is attenuated by administration of DOXY pre HI (C), 1 hour post HI (D), and 3 hours post HI (E). SHAM animal is shown in (A). Scale bar = 100μm

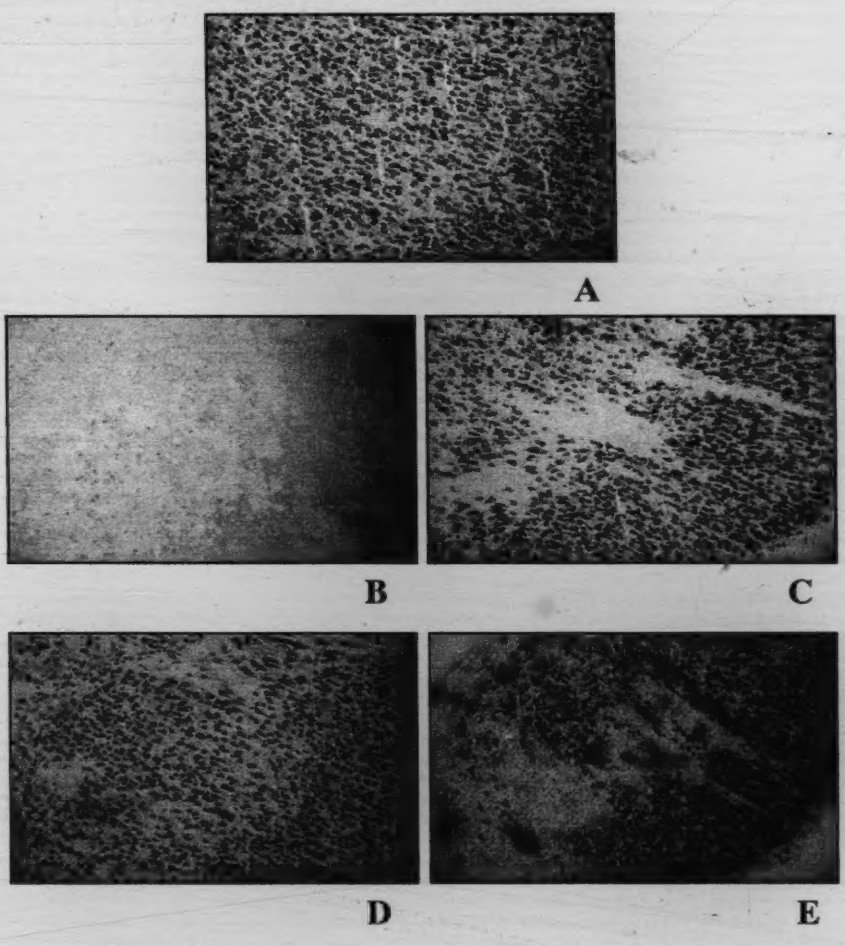


Figure 3.4. Neuronal Cell Loss in the Cortex After Moderate Hypoxia-Ischemia. Representative photomicrographs illustrating the pattern of cell loss, cystic-like lesions and cortical infarction typical of vehicle-treated HI rat pups (B) is attenuated by administration of DOXY pre HI (C), 1 hour post HI (D), and 3 hours post HI (E). SHAM animal is shown in (A). Scale bar = 50μm.

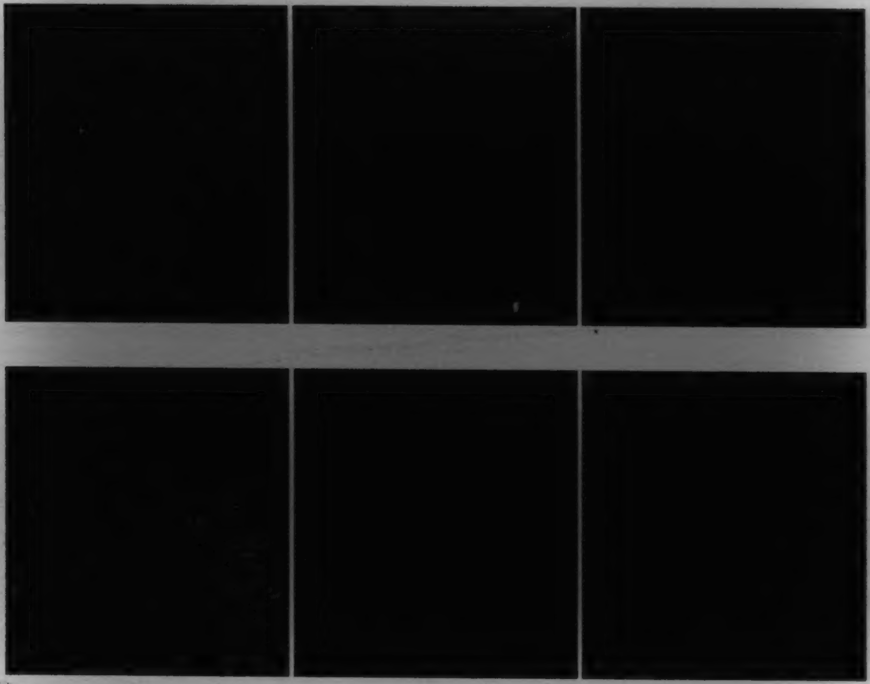


Figure 3.5. Fluoro-Jade Staining For Degenerating Cells in the Cortex and Lateral Hippocampus After Moderate Hypoxia-Ischemia (HI). A moderate hypoxic-ischemic brain injury results in numerous degenerating cells throughout the hippocampal formation and dorsal lateral cortex of VEH-treated pups (B). Enlargement of the lateral ventricles are also observed (B. D). Neuronal degeneration is partially attenuated by administration of DOXY pre HI (C), 1 hour post HI (D), and 2 hours post HI. However, the administration of DOXY does not significantly reduce the number of degenerating cells when administered 3 hours after a moderate HI injury (F), as countless Fluoro-jade positive cells surrounding infarcts are observed. SHAM animal is shown in (A). Scale bar = 100μm.

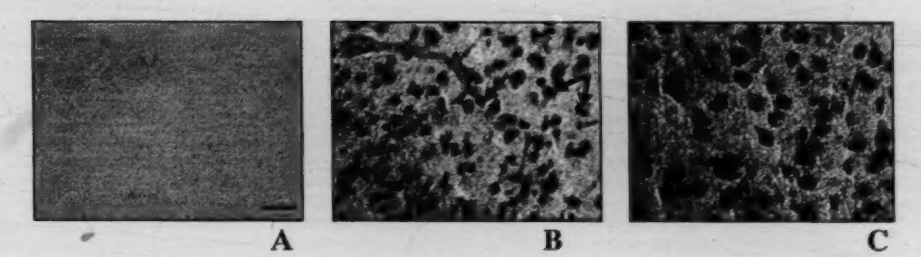


Figure 3.6. Cleaved Caspase-3 Immunoreactivity. Cleaved caspase-3 immunoractivity is different in the ipsilateral (B) and contralateral (C) sides of the neonatal brain subjected to hypoxia-ischemia. Representative photomicrographs of cleaved caspase-3 immunopositive cells in the cerebral cortex of postnatal day (P) 10 rat brains shown 3 days after common carotid ligation and exposure to hypoxia for 1h at P7. The right cerebral cortex (B) has significantly more immunopositive cleaved caspase-3 cells (arrows) as compared to the contralateral, left cortex (C). Section from right cortex incubated with cleaved caspase-3 antibody and a specific blocking peptide is shown in (A). Scale bar = 10μm.

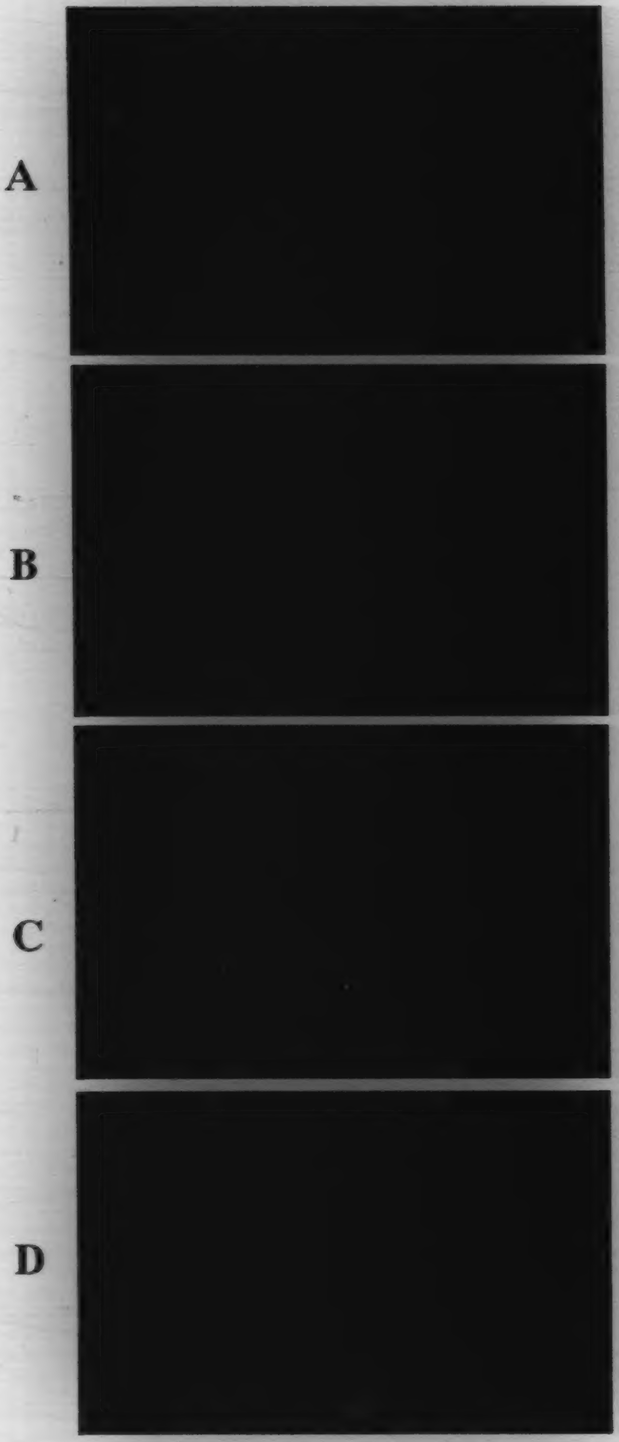
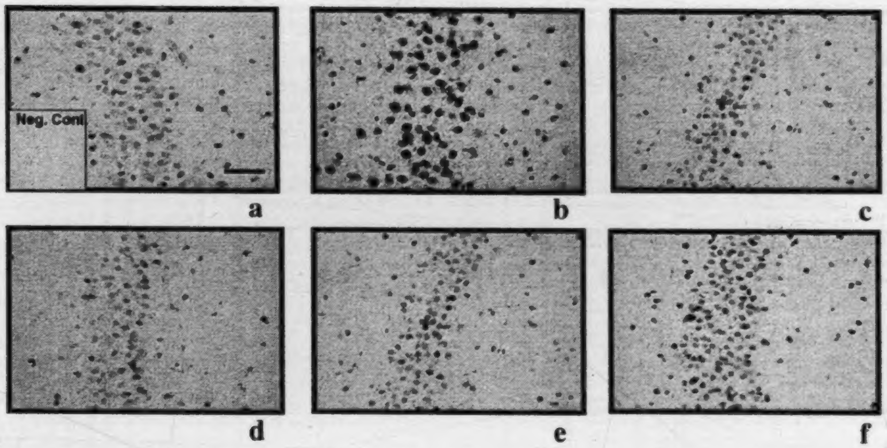

A
B
C
D

Figure 3.7. Neurons and Oligodendrocytes are Positive for Cleaved Caspase-3. Double immunostaining for (A) NeuN (neurons, green) and (B) cleaved caspase-3 (red) in the hippocampus of hypoxic-ischemic rat pups. Plate (C) represents the overlay of images (A) and (B), where neurons that are positive for cleaved caspase-3 appear yellow/orange. Notice the red caspase-3 positive cells in the white matter dorsal to the hippocampus in both (B) and (C). These caspase-3 positive cells are not neurons; they were shown to be oligodendrocytes (D). Oligodendrocytes that are positive for cleaved caspase – 3 appear yellow/orange. Scale bar = 20μm.

A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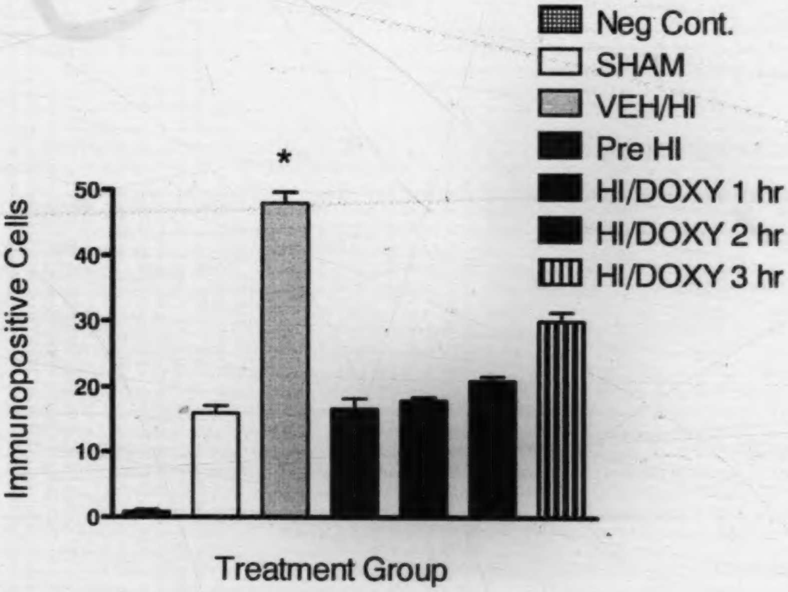
B


▦ Neg Cont.
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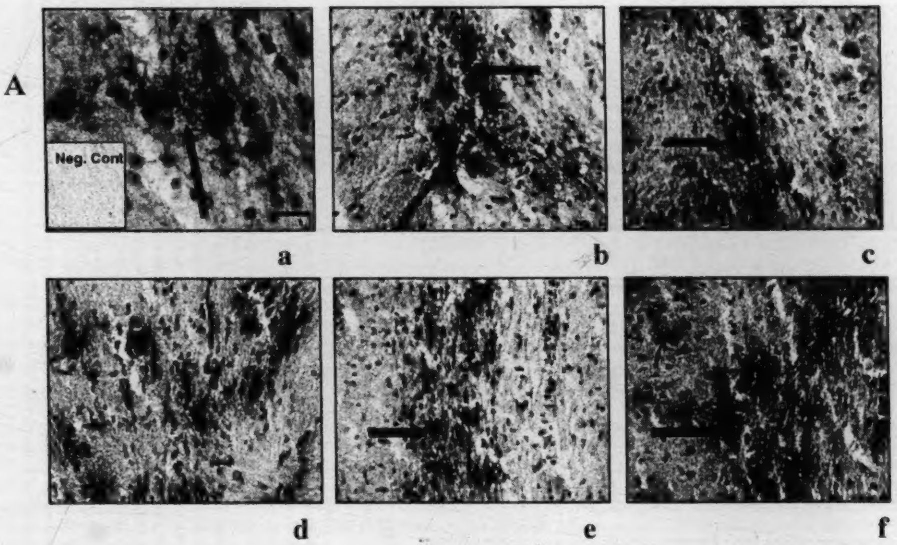
Immunopositive Cells
0
10
20
30
40
50
Treatment Group

Figure 3.8A. Cleaved Caspase-3 Immunoreactivity in Mild Hypoxia-Ischemia (HI) In Response To Treatment with Doxycycline. Doxycycline decreases cleaved caspase-3 immunoreactivity in the neonatal brain subjected to mild hypoxic-ischemic injury. Representative photomicrographs of hippocampal sections (CA1) of postnatal day (P) 14 rat brains shown 1 week after carotid ligation and exposure to hypoxia for 1h at P7. Basal level of caspase-3 immunoreactivity shown in SHAM animals (a) is significantly less than that observed in vehicle- treated hypoxic-ischemic pups (b). In typical sections from animals administered intraperitoneal injections immediately before hypoxia (c), 1 hour post hypoxia (d), 2 hours post hypoxia (e) and 3 hours post hypoxia (f), doxycycline decreased immunoreactivity. Scale bar = 20μm.

Figure 3.8B. Cleaved Caspase-3 Immunopositive Cells in the Hippocampus After Mild Hypoxia-Ischemia (HI). Bars represent the mean (± standard deviation) field values for each group. Vehicle-treated hypoxic-ischemic rat pups have significantly more cleaved caspase-3 immunopositve cells compared to the SHAM animals. DOXY- treated pups were not significantly different from the SHAM controls (ANOVA, Newman-Keul *post hoc* analysis, $p<0.05$).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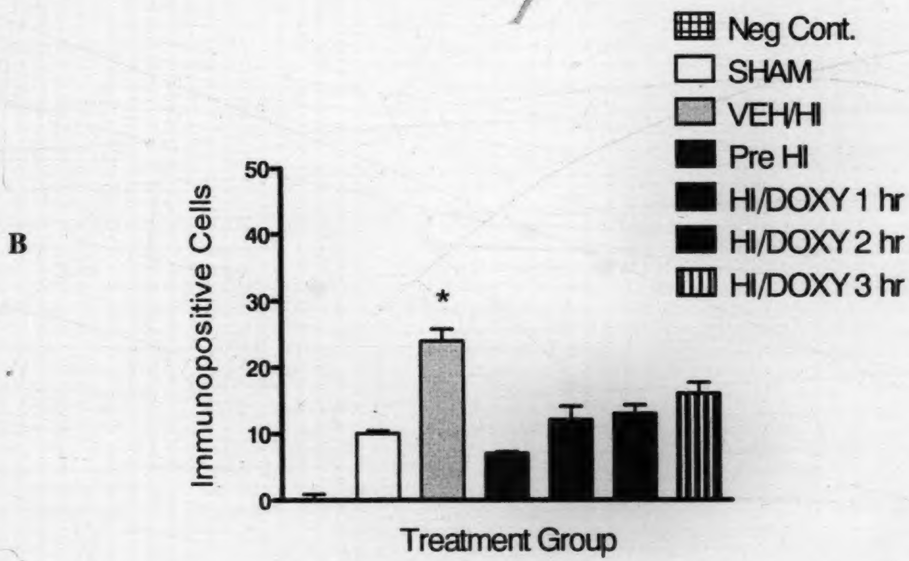
B
Neg Cont.
SHAM
VEH/HI
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HI/DOXY 1 hr
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Immunopositive Cells
50
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0
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Treatment Group

Figure 3.9A. ED-1 Immunoreactivity in Mild Hypoxia-Ischemia (HI). Doxycycline decreases ED-1 immunoreactivity in the neonatal brain subjected to mild hypoxic-ischemic injury. Representative photomicrographs of hippocampal sections of postnatal day (P) 14 rat brains shown 1 week after carotid ligation and exposure to hypoxia for 1h at P7. Basal levels of ED-1 immunoreactivity in sham animals (a) show mainly rod-shaped, resting microglia. Significantly more round, activated microglia (arrows) and increased ED-1 immunoreactivity were present in vehicle treated HI pups (b). Administration of doxycycline decreases ED-1 immunoreactivity compared with the VEH-treated HI pups. Plate (c) DOXY administered pre-hypoxia, (d) DOXY administered 1 hour post HI, (e) DOXY administered 2 hours post HI and (f) DOXY administered 3 hours post HI [plate (a) scale bar = 10μm; (b-f) scale bar = 20μm].

Figure 3.9B. ED-1 Immunopositive Cells in the Hippocampus After Mild Hypoxia-Ischemia (HI). Bars represent the mean (± standard deviation) field values for each group. Vehicle treated hypoxic-ischemic rat pups have significantly more ED-1 immunopositive cells compared to the SHAM animals. DOXY-treated rat pups were not significantly different from the sham controls (ANOVA, Newman-Keul *post hoc* analysis, $p<0.05$).

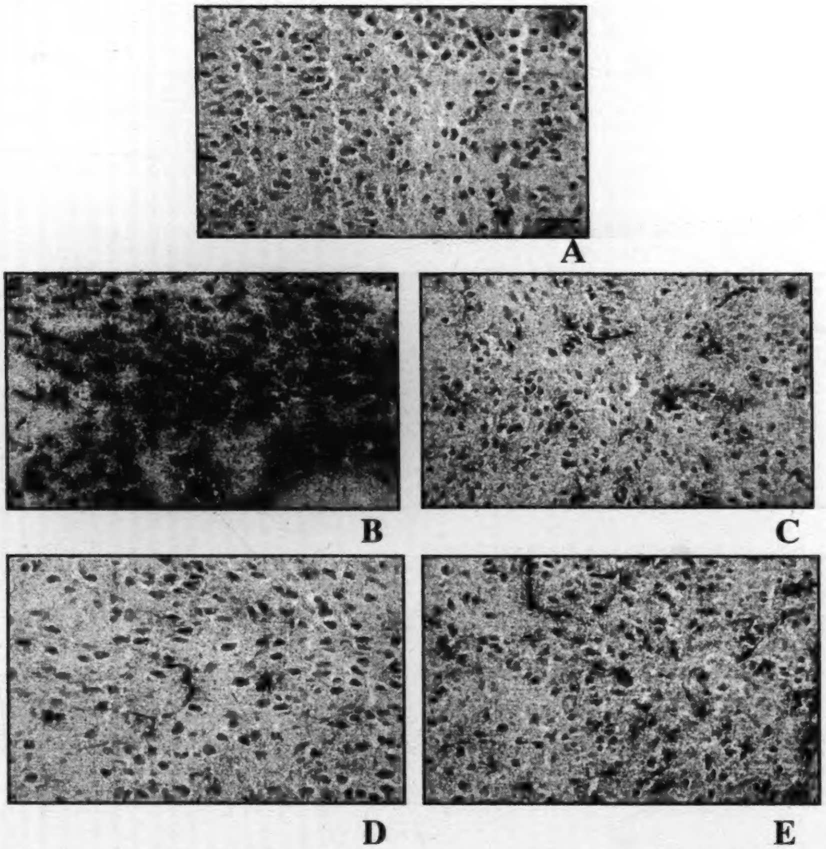

Figure 3.10. ED-1 Immunoreactivity In Moderate Hypoxia-Ischemia (HI). Doxycycline decreases ED-1 immunoreactivity in the neonatal brain subjected to moderate hypoxic-ischemic injury. Representative photomicrographs of the thalamus of postnatal day (P) 14 rat brains shown 1 week after carotid ligation and exposure to hypoxia for 1.5h at P7. Basal levels of ED-1 immunoreactivity in sham animals are shown in (A). Significantly more round, activated microglia and increased ED-1 immunoreactivity are present in vehicle treated HI pups (B). Administration of doxycycline decreases ED-1 immunoreactivity compared with the VEH-treated HI pups. Plate (C) doxycycline administered pre-hypoxia; (D) doxycycline administered 1 hour post HI; and (E) doxycycline administered 3 hours post HI. Scale bar = 20μm.

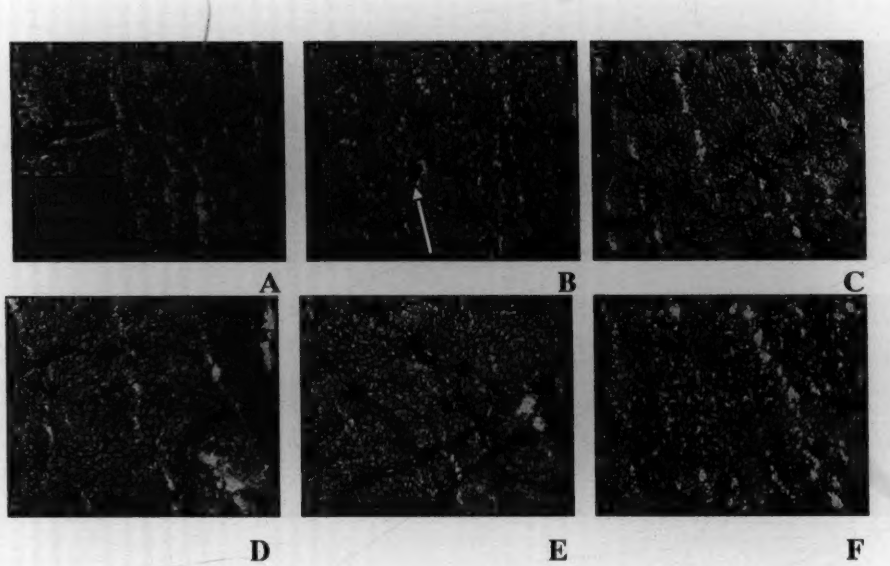

Figure 3.11. Doxycycline Changes the Morphology of Astrocytes in Neonatal Rats Subjected to Mild Hypoxia-Ischemia (HI). GFAP positive astrocytes were present in all experimental and control animals. The SHAM animals (A) exhibited basal levels of GFAP expression manifested by delicate-looking astrocytes with relatively small cell bodies and thin projections. Increased expression of GFAP and increased cell body size causing hypertrophy (arrow) was observed in VEH-treated HI pups (B). DOXY administration resulted in GFAP-positive cells with smaller cell bodies and thinner projections compared to the VEH-treated HI pups. Plate (C) DOXY administered pre hypoxia; (D) 1 hour post HI; (E) 2 hours post HI; (F) 3 hours post HI. Scale bar = 20μm.

3.5. References

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CHAPTER 4

The Consequences of Doxycycline Administration on Cell Genesis After Neonatal Hypoxia-Ischemia: A Timecourse Evaluation

4.1. Introduction

The previous investigations described in this thesis provided crucial information on the acute and sub-acute neuroprotective and anti-inflammatory properties of doxycycline. Further, data were collected to suggest that DOXY could no longer be thought of as strictly an anti-inflammatory or anti-apoptotic drug, as related to the modulation of amino acids. Given that inflammation, apoptosis and amino acid neurotransmitters have roles in both brain development and the pathology of neonatal HI, further investigation into the consequence of inhibiting these processes was required. As such, the purpose of this chapter was to investigate the consequences of DOXY administration in neonatal HI related to the birth of new cells and neurogenesis. To this end, a timecourse evaluation of the putative impact of DOXY administration on cell proliferation and differentiation was performed.

Recent studies show that neurogenesis occurs in two major prolifatory regions of the mammalian brain, the dentate gyrus (DG) and the subventricular zone (SVZ). In the DG, newly generated neuronal cells in the subgranular layer migrate to the granular layer (Taupin 2007, von Bohlen und Halbach 2007), while newborn cells in the anterior part of the SVZ migrate through the rostro-migratory stream to the olfactory bulb (Lois & Alvarez-Buylla 1994, Taupin 2007). However, under pathological conditions in the neonate, it has been reported that the division of cells in these zones can be enhanced and that new cells may be integrated into the existing neuronal network and participate in recovery after injury (Hayashi *et al.* 2005, Ong *et al.* 2005, Parent *et al.* 2002, Arvidsson *et al.* 2002, Plane *et al.* 2004). In general, neurogenesis decreases with stress and age and it has been well documented that exposure to alcohol, opiates and prenatal viral infections also causes decreases in neurogenesis. Increased neurogenesis has been documented in relation to environmental enrichment, reduced caloric intake, physical activity and as already mentioned, pathological insults such as seizure and stroke (Parent *et al.* 2002, Parent *et al.* 1997). Many studies of neonatal HI are concerned with how cell death can be prevented or at least minimized (Chang *et al.* 2006). However, the neonatal brain possesses a larger ability to produce new cells as compared to the adult, and it is now believed that neurogenesis could be exploited to improve recovery post-HI (Hayashi *et al.* 2005, Vaccarino & Ment 2004).

The full spectrum of microglial activity imparts complex outcomes on the integrity of the CNS (Nuttall *et al.* 2007). While persistent microglial activation and the secretion of a range of molecules are thought to be detrimental, some microglial responses such as the production of neurotrophic factors and removal of toxic products serve to limit injury and enhance repair (Nuttall *et al.* 2007, Lai & Todd 2006b, Lai & Todd 2006a, Lai & Todd 2008, Batchelor *et al.* 1999, Simard *et al.* 2006). For example, microglia can enhance neural stem cell proliferation and the subsequent migration of new cells to injured areas of the brain through the excretion of diffusible factors (Aarum *et al.* 2003). On the contrary, microglial activation associated with inflammation has been shown to impair both basal and insult-induced neurogenesis, with significant negative correlations existing between numbers of activated microglia and numbers of newborn neurons (Ekdahl *et al.* 2003). As we have previously reported that DOXY significantly inhibits microglial activation after neonatal HI, whilst improving neuronal survival and decreasing cleaved caspase-3 protein expression, the goal of this series of experiments was to evaluate the consequences of DOXY administration specifically related to cell genesis over a 7 day timecourse post-HI. As well, we sought to identify the phenotype of the progeny of new cells in relation to HI alone, and treatment with DOXY.

4.2. Materials and Methods

4.2.1. Animals and Surgical Procedures

As previously described in earlier chapters, we used a well characterized procedure combining unilateral common carotid occlusion with systemic hypoxia to produce HI brain injury (Jantzie *et al.* 2005, Jantzie *et al.* 2006). The paradigm employed produces unilateral brain damage as a consequence of acute reduction in blood flow and oxygenation to mimic the disruption in nutrient delivery that is the primary cause of neurological injury during the perinatal period (Yang & Levison 2007). As with all animal experiments performed, the procedures described are in accordance with the guidelines set forth by the Canadian Council on Animal Care and were approved by the Health Sciences Animal Policy and Welfare Committee of University of Alberta.

4.2.2. Drug Administration and Tissue Preparation

DOXY (SIGMA, 10 mg/kg) or saline vehicle (VEH) was administered intraperitoneally in a volume of 100 μl as a one-time dose immediately before HI.

Pups were then euthanized by decapitation 3h, 6h, 12h, 24h, 48h or 7days after the HI insult (n=6). To label proliferating cells, pups received 5-bromo-2-deoxyuridine intraperitoneally (BrdU, SIGMA, 30mg/kg) concomitant with the administration of DOXY/VEH. Pups surviving 12-48h post-HI received additional doses of BrdU every 12h. Pups enrolled in the sub-acute survival portion of this neurogenesis investigation (7 day survival) did not receive BrdU on P7, but rather received BrdU beginning on P12 and were dosed every 12h until euthanasia on P14. The number of cells labeled by a single injection of BrdU is limited by the bioavailability of BrdU (half-life = 2h) and the number of cells in S-phase during this brief period (i.e. the number of S-phase cells during the first 2 hours after the injection). Thus, when interested in marking the entire population of newly formed cells during a time span, repeated pulses of BrdU are required (Gilbert *et al.* 2005). It was in light of this information that the BrdU dosing regimen described above was undertaken. Upon euthanasia, brains were removed and flash frozen in isopentane on dry ice. These samples were stored at –80 °C until analysis.

4.2.3. Immunohistochemistry

For immunohistochemcial analyses, serial coronal sections (20 μm) throughout the brain were obtained using a cryostat. Immunostaining was performed using antibodies recognizing neurons (Alexa-Fluor® 488 anti-neuronal nuclei [NeuN], Chemicon, 1:100), cleaved caspase-3 (rabbit anti-cleaved caspase-3, Cell Signaling Technology, 1:1000), BrdU (mouse anti-Brdu, Roche, 1:600), nestin (mouse anti-nestin, Chemicon, 1:100), proliferating cells (mouse anti-Ki-67, Dako, 1:100), activated microglia (mouse anti-ED-1, Serotec, 1:100) and astrocytes (Alexa-Fluor® 488 anti-glial fibrillary acidic protein [GFAP], Chemicon, 1:500). For each animal, immediately adjacent sections were used for each of the primary antibodies. Briefly, fresh frozen sections were post-fixed in buffered formalin and then defatted and dehydrated in a graded series of ethanol washes. Sections were then incubated in a humidifying chamber with 0.03% hydrogen peroxide and were subsequently blocked with 10% Normal Horse Serum and 0.5% Triton-X 100 in phosphate buffered saline (PBS). Sections were allowed to incubate with the primary antibodies overnight. After incubation with the species appropriate fluorochrome-tagged secondary IgG antibody (all Jackson, 1:100) for 30 min the sections were carefully washed and coverslipped with an aqueous mounting media (Sigma).

Those sections being immunostained for or with BrdU underwent a DNA denaturation step in 2M HCl for 60 min at 37.5 °C immediately after fixation. These sections were then neutralized in 0.1M sodium borate (2 X 5min) and PBS before proceeding with the hydrogen peroxide step and carrying on with the rest of the immunohistochemical procedure.

Double and triple immunostaining was also performed in accordance with the procedures described above, with serial applications of primary antibodies and species appropriate, fluorescent-tagged secondary antibodies. For example, mouse anti-BrdU (1:600) was applied to the sections and allowed to incubate overnight at 4°C. The following day, after judicious washing with PBS, anti-mouse IgG conjugated to Texas Red (1:100) was applied to the sections for 30 min. Sections were then washed and returned to 4°C for overnight incubation with rabbit anti-cleaved caspase-3 (1:1000). The next day, sections were either washed and coverslipped or incubated with Alexa-Fluor® 488 anti-neuronal nuclei (NeuN) for a minimum of 2h. For the triple labeled sections, final rinses with PBS and ddH_20 were then performed and sections were coverslipped with an aqueous mounting media (Sigma).

4.2.4. Quantification of 5-bromo-2-deoxyuridine (BrdU)-Positive Cells

The numbers of BrdU-positive cells were counted in 6 animals at each time-point and each treatment group, in each of the medial SVZ and DG. To perform cell counts, locations were identified using specific landmarks and in reference coronal plate #16 (SVZ) and #30 (DG) in Paxinos & Watson (Figure 4.1) (Paxinos & Watson 1998). Under the 10x microscope objective a photomicrograph was taken of the designated fields by an investigator blind to the treatment groups and end points. All BrdU-positive nuclei in these designated areas were then counted and presented as numbers of cells (in hundreds). A second investigator blind to all conditions also performed cell counts on a sub-set of randomly selected sections to conform to standard inter-judge reliability practices.

4.2.5. Statistical Analyses

All results shown are expressed as mean ± standard deviation. Data were analyzed by one-way ANOVA followed by the Newman-Keuls multiple comparison test on significant main effects and interactions. Where appropriate, unpaired t-tests were used and when variances between two groups were significantly different a Welch's correction was employed. The general convention of a probability value of $p<0.05$ was used to establish statistical significance.

4.3 Results

4.3.1. 5-bromo-2-deoxyuridine (BrdU) Immunoreactivity

BrdU immunoreactivity was seen throughout the neonatal rat brain, in both SHAM and HI animals, at all points along the timecourse. BrdU-positive cells were clearly identifiable as bright illuminations above background, and the staining of these cells was observed as nuclear and often punctate. Although numerous BrdU-positive cells were seen in the cortex and thalamus, the majority of BrdU-positive cells were concentrated in the SVZ and DG (Figures 4.2-4.5). Because BrdU immunoreactivity was so intense and numerous, antibody specificity was determined by examining the amount of BrdU immunoreactivity in naïve adult animals (Figure 4.2A). As well, BrdU immunoreactivity was compared to Ki-67 immunoreactivity (Figure 4.2C), as Ki-67 is a marker of proliferating cells in G1, S, G2, and mitosis. In the naïve adult, sparse BrdU immunoreactivity was localized solely to the dorsal SVZ. As expected, P7 rat pups had significantly more BrdU positive cells throughout the brain compared to naïve adults (Figure 4.2B). Ki-67 immunostaining was more numerous and corroborated BrdU immunoreactivity (Figures 4.2B, 4.2C).

As the major zones of proliferation and sites of cell birth, the numbers of BrdU-positive cells were quantified in the SVZ and DG (Figures 4.3-4.6). Analyses throughout the timecourse revealed that treatment with DOXY significantly decreased the number of BrdU-positive cells in the SVZ, 6 hours post-HI as compared to VEH-treated HI pups (Figures 4.3-4.4). Further, SHAM animals treated with DOXY (SD) also had significantly fewer BrdU-positive cells in the SVZ compared to SHAM animals, at 6 hours (Figure 4.4). Twenty-four hours post-HI, DOXY-treated HI pups also had fewer BrdU-positive cells in the SVZ although this decrease failed to reach statistical significance (p=0.0572). The complete timecourse of BrdU immunoreactivity shown in Figure 4.4 clearly depicts the biphasic decrease in the number of BrdU positive cells occurring at 6 and 24h. However, by 48h and with extension to 7d post-HI, there is no sustained change in the number of BrdU positive cells in any experimental group (Figure 4.4).

The typical pattern and distribution of BrdU immunoreactivity in the DG is depicted in Figures 4.5 and 4.6. Quantification analyses reveal that at no time point investigated do HI rat pups treated with DOXY have significantly different number of BrdU positive cells as compared to VEH-treated HI pups (Figure 4.6). However, 6 hours post-HI, DOXY treated pups have significantly fewer BrdU positive cells as compared to SHAM animals (Figure 4.6). DOXY-treated pups have fewer BrdU positive cells in the DG as compared with VEH-treated pups, 12 hours post-HI although this is not statistically significant (p> 0.05). The complete timecourse of BrdU immunoreactivity displayed in Figure 4.6 clearly depicts the steady increase in BrdU-positive cells peaking 48h post-HI and decreasing by 7-days post injury.

4.3.2. Identifying the Phenotype of the Progeny

The next step in this investigation was to determine the phenotype of the numerous new cells. Based on morphology and location in the SVZ and DG it was hypothesized that the majority of new cells were or would become, of neuronal phenotype. Depending on the point along the time course examined, very few BrdU-positive cells co-localized with an antibody against neuronal neuclei (NeuN), a marker of post-mitotic neurons (Figure 4.7). From 3-48h there were no identifiable co-localizations, but by 7days a few cells in both the SVZ and DG displaying BrdU and NeuN colocalization were observed (data not shown, but is observable in Figure 4.11). To identify cell type, co-localization experiments with GFAP (Figure 4.8), ED-1 (data not shown) and nestin (a marker of immature multi-potent precursors) were also performed. Throughout the timecourse, it was found that the majority of BrdU-positive cells did not co-localize with GFAP (Figure 4.8) or ED-1, although it was noted that more co-localization with GFAP occurred in the earlier points along the timecourse (i.e. 3-6h post HI, data not shown). Figure 4.8 is representative of the typical pattern of BrdU and GFAP double immunostaining.

4.3.3. Identifying New Cells vs. Cells Re-entering the Cell Cycle

To conclude that the BrdU cells examined and quantified in this investigation were in fact "new" and not simply damaged cells re-entering the cell cycle as part of neurodegeneration post-HI, an absence of cell death markers in the BrdU positive cells was demonstrated. Figures 4.9 and 4.10 depict the changes in BrdU/Cleaved Caspase-3 immunoreactivity throughout the timecourse investigated. Acutely after

HI, very few BrdU/Cleaved caspase-3 positive cells were identified in either the SVZ (Figure 4.9) or DG (data not shown). Seven days post-HI there were significantly more cleaved caspase-3 positive cells in general, and more of these cells were found co-localized with BrdU in the SVZ (Figure 4.9). However, as clearly demonstrated in Figures 4.9 and 4.10, the majority of BrdU positive cells in the proliferative zones do not co-localize with cleaved caspase-3. This observation is consistent throughout the timecourse investigated. Figure 4.11 depicts triple labeling performed with antibodies against BrdU, NeuN and cleaved caspase-3, 7d post-HI. As a few BrdU-positive cells were identified to co-localize with NeuN at the latest point in the timecourse, triple labeling was performed in an attempt to identify if any new cells of neuronal phenotype were also expressing a cell death marker. As depicted in Figure 4.11, numerous NeuN-positive cells (green), and cleaved caspase-3 cells (blue) exist on their own in the SVZ and surrounding striatum. Additionally, BrdU/NeuN positive cells (yellow/orange) and BrdU/Cleaved caspase-3 positive cells (purple) were observed in this region. The majority of BrdU/NeuN positive cells are negative for triple labeling with cleaved caspase-3. This pattern of immunostaining was consistent and replicated in the DG (data not shown).

4.3.4. Nestin Immunoreactivity

Immunostaining was performed for nestin, as nestin is present in neural-specific intermediate filament proteins and can also be expressed by astrocytes and radial glial during development. The typical pattern and distribution of nestin-positive cells in the SVZ is shown in Figure 4.12A. Co-localization experiments reveal that the majority of nestin positive cells in SVZ are negative for GFAP (Figures 4.12B, 4.12C). Sporadic nestin/GFAP co-localization is observed medially, as depicted in Figure 4.12C and 4.12D.

4.4. Discussion

The results of this investigation provide insight into the consequences of DOXY administration on neurogenesis and the birth of new cells post-HI. For the first time, evidence is presented indicating that DOXY acutely, but not persistently, inhibits cell genesis (as indicated by BrdU immunoreactivity) in both the SVZ and DG. Further it is demonstrated that the newborn cells, a small proportion of which are of mature neuronal phenotype, exist independently of cell death markers.

Numerous BrdU-positive cells were found throughout the neonatal brain in both SHAM and HI animals, with the highest density of BrdU-positive cells localized to the proliferative zones of the SVZ and DG. The number and distribution of BrdU positive cells was observed as expected considering the age and developmental stage of the pups. As mentioned in the general introduction, BrdU is a deoxythymidine analogue and is incorporated into new DNA during the S-phase of the cell cycle. Thus, BrdU is a marker of DNA synthesis and not a *direct* indicator of cell proliferation as it may also label cells undergoing DNA repair, abortive cell cycle reentry, as a prelude to apoptosis, and gene duplication without cell division (Taupin 2007).

The numbers of BrdU-positive cells variably increased and decreased in the SVZ and DG throughout the timecourse post-HI. The specificity of the BrdU immunoreactivity was confirmed not only with evaluation of the numbers of BrdU-positive cells in naïve adult animals, but also by comparison to Ki-67 immunostaining in neonatal animals. In naïve adults, BrdU immunoreactivity was localized only to the most dorsal aspect of the SVZ as shown in Figure 4.2A, with sparse immunoreactivity in the subgranular zone in the DG (data not shown). As expected, there were significantly fewer BrdU-positive cells in adult animals compared with the neonates. As Ki-67 is expressed in all phases of the cell cycle except the resting stage, Ki-67 immunoreactivity was compared to BrdU immunoreactivity to directly assess cell proliferation. Ki-67 has a very short half-life, is not detectable during DNA repair processes and is strongly downregulated/absent in quiescent cells (Zacchetti *et al.* 2003). As expected, this comparison revealed that there were significantly more Ki-67 cells compared with BrdU-positive cells throughout the neonatal brain, especially in the SVZ. These data corroborated the documented BrdU immunoreactivity. Disruption of the BBB and/or alterations in cerebral blood flow could potentially increase BrdU labeling as a result of increased BrdU uptake rather than real changes in cell proliferation and neurogenesis (Gould & Gross 2002, Taupin 2007). As BBB breakdown appears to alter BrdU availability with the potential to change quantitative data, and because the BBB is not completely formed in immature animals (complete formation begins around P10), Ki-67 immunostaining validated our cell proliferation assessments as an independent indicator of stem cell proliferation and neurogenesis.

Upon further analysis of BrdU immunoreactivity in HI pups, it was found that neither DOXY-treated nor VEH-treated pups had any significant increases or decreases in the number of BrdU-positive cells compared to SHAM animals in the SVZ. This trend was maintained in the DG, although at 6 hours post-HI, DOXY-treated pups had significantly fewer BrdU-positive cells as compared to SHAM. In previous investigations, acute reductions in BrdU immunoreactivity and a depletion of neural progenitors have been demonstrated following moderate HI (Levison *et al.* 2001). These findings were not replicated in this study, as we incorporated a lesioning protocol that elicited a relatively mild HI injury. This was purposeful as we titrated the HI injury such that significant portions of the SVZ/striatum and DG/hippocampus were not infarcted or directly damaged. In relation to mild HI injuries, it is highly unlikely that our findings represent changes in cell density due to surrounding tissue loss as opposed to changes in the absolute numbers of new cells present (Bartley *et al.* 2005). When examining the compilation histograms in Figures 4.4 and 4.6, the change in the number of BrdU-positive cells over the timecourse post-HI is clearly demonstrated. In the SVZ, the number of BrdU-positive cells peaked at 12-24h post-HI and in the DG, the peak of BrdU immunoreactivity occurred at 48h. In both regions, the number of BrdU positive cells significantly decreased in both VEH- and DOXY-treated pups 7d post-HI (P14) in accordance with the expected reduction in cell genesis with age.

As alluded to before, successful BrdU integration requires extensive optimization of experimental procedures. BrdU is toxic and mutagenic, with side effects related to the integration of a bromine atom into DNA (Taupin 2007). These side effects change DNA stability and increase the risk of sister-chromatid exchanges, and DNA double strand breaks, whilst lengthening the cell cycle of those that incorporate it. Further, high doses of BrdU (60-600 mg/kg) can directly trigger neuronal cell death during neonatal development, although lower doses have not shown toxic effects on the development of the SVZ (Takahashi *et al.* 1995). Mitochondrial DNA can also incorporate BrdU at high doses (Cooper-Kuhn & Kuhn 2002). In light of this knowledge, the lowest concentration of BrdU required to obtain effective cell labeling was used in this investigation. This dose was titrated and optimized at 30mg/kg.

The DNA denaturation process that involves partial tissue hydrolysis with HCl is crucial for successful BrdU immunostaining. However, this pretreatment affects morphology and antigenic recognition in multiple labeling studies, thereby limiting the morphological and phenotypical identification of newly generated cells (Taupin 2007). Despite these inherent issues with methodology, we were successful in providing evidence that the phenotype of a small proportion of new, BrdU-positive cells were of mature neuronal phenotype. It is duly recognized that the maturation of newborn cells, from proliferation in the subgranular zone to migration and differentiation in the granular layer, takes approximately 4 weeks in the adult rat DG (Cameron *et al.* 1993). However, we observed sporadic BrdU/NeuN co-localization at our latest timepoint, 7d post-HI. In the more acute time points investigated, there was no co-localization with NeuN, as indicated in Figure 4.7. However, we did find many "satellite cells", or BrdU-positive cells closely apposed to mature, NeuN-positive neurons as documented by other investigators (Ong *et al.* 2005, Kuhn *et al.* 1997). This finding was not unexpected, as NeuN is a soluble nuclear protein localized to the nucleus and in the neuronal cytoplasm of post-mitotic neurons (von Bohlen und Halbach 2007). Thus, expression of this antigen first appears at developmental time points corresponding with withdrawal of the neuron from the cell cycle and/or with initiation of terminal differentiation (Mullen *et al.* 1992, Lind *et al.* 2005). In contrast, nestin is a marker of neural progenitor and stem cells. During brain development, nestin is expressed by astrocytes and radial glia, and as the brain matures, the expression of nestin wanes. Additionally, GFAP is widely accepted as a marker for mature astrocytes, although a large proportion of newborn cells can also be GFAP-positive (von Bohlen und Halbach 2007). In our investigation, the phenotypic identification of newly generated cells at the time of euthanasia was performed by multiple labeling, against BrdU, GFAP, as well as immature and mature neuronal markers. It was found that the younger the pup (i.e. the shorter survival after HI), the more BrdU/GFAP and BrdU/ED-1 positive cells were present. However, as depicted in Figure 4.8, the majority of BrdU positive cells did not co-localize with GFAP from 24h post-HI onward. Numerous nestin-positive cells were identified in the SVZ post-HI, and these cells showed sparse co-localization with GFAP. Although the BrdU-positive cells in our study were co-labeled with antibodies recognizing different types and sub-types of cells, there were many BrdU-positive cells that seemed to be in an intermediate stage of development. It is probable that these are immature, undifferentiated cells or uncommitted neural precursor cells not yet expressing the markers we evaluated (Bartley *et al.* 2005). This result could be attributed to the BrdU labeling protocol used that consisted of BrdU injections over the course of the experiment, leading to the labeling of more cells than would be found following a single injection. It is also possible that the injured brain in this stage of development does not provide an environment that is conducive to the maturation of newly born neurons. Although the explanation that these cells are uncommitted neural precursors is most likely, the only way to know conclusively would be to examine the phenotype of these cells with additional cell markers under a much longer timecourse. This being said, we ruled out the possibility that the majority of these cells were astrocytes or microglia (data not shown). Further, other investigators have shown that many BrdU-positive cells acutely following HI are endothelial cells and oligodendrocytes but that gliogenesis significantly declines with age. Thus, in light of this information and because of our data that show numerous nestin-positive cells throughout the brain and sporadic NeuN immunoreactivity, we deduce that the majority of the cells examined are neurons in an intermediate developmental stage.

Many new neurons fail to become established in the existing neuronal network after proliferation and migration, and there is increased evidence that acute neural injury triggers continuing cell loss as related to chronic encephalopathic processes (Barrett *et al.* 2007). The precise variables that hinder new neurons from becoming established are unknown; however local environmental factors such as inflammatory cytokines, activated microglia, astrogliosis, and disruption of trophic support are likely important (Cai *et al.* 2006, Chew *et al.* 2006, Barrett *et al.* 2007). When studying neurogenesis, it is absolutely crucial to demonstrate not only BrdU uptake in the presence of mature neuronal markers but also to provide evidence showing an absence of apoptotic markers (Kuan *et al.* 2004). This is vital, especially in neonatal HI, as a recent finding suggests that HI preferentially triggers neurons to re-enter the cell cycle and resume apoptosis-associated DNA synthesis (Kuan *et al.* 2004). We characterized the number of BrdU/Cleaved caspase-3 positive cells initially present post-HI. As depicted in Figure 4.9, very few BrdU positive cells in the SVZ co-localized with cleaved caspase-3, an end effector of caspase-dependent apoptotic cell death. Seven-days post-HI, there were more BrdU/cleaved caspase-3 positive cells as compared to the earlier timepoints. This increase in the number of BrdU/cleaved caspase-3 positive cells observed as time elapsed is consistent with the evolution of cell death post-HI injury as demonstrated in earlier chapters. Given these data, we then demonstrated that the majority of BrdU-positive cells in both the SVZ and DG remained negative for a co-localization with cleaved caspase-3, throughout the 7d survival period. To corroborate, the triple labeling performed demonstrated BrdU colocalization with a mature neuronal marker (NeuN), in the absence of cleaved caspase-3.

In conclusion, administration of DOXY significantly decreases the number of BrdU-positive cells in the SVZ, acutely following HI. However, treatment with DOXY does not persistently affect the number of BrdU positive cells in either the SVZ or DG as evidenced by the consistent numbers of BrdU positive cells 48h-7d post-HI, in either treatment group. This reduction in BrdU immunoreactivity is almost certainly related to DOXY itself, as opposed to an epiphenomenon related to HI, as it was observed in both the treated-SHAM and the HI pups (Figure 4.4). Although the mechanisms for this is unclear at this point and will be investigated in the next chapter, it is postulated that the decrease in BrdU-positive cells reflects and corresponds to peak DOXY levels in the brain and thus, maximal microglial inhibition. In support of this hypothesis, other studies have shown that microglia selectively promote the survival of neuronal cells (i.e. by providing neurotrophins) and may be involved in neuroblast migration. Although we were not able to demonstrate numerous newly generated cells expressing mature neuronal cell markers, the lack of co-localization with GFAP and the large proportion of nestin immunoreactivity suggest that these cells are not yet differentiated but likely on their way to becoming neurons. Further, where co-localization between BrdU and NeuN existed there was an absence of triple-localization with cleaved caspase-3, indicating that these were indeed new neurons (i.e. neurogenesis had occurred) and not neurons re-entering the cell cycle as part of neurodegeneration post-HI.

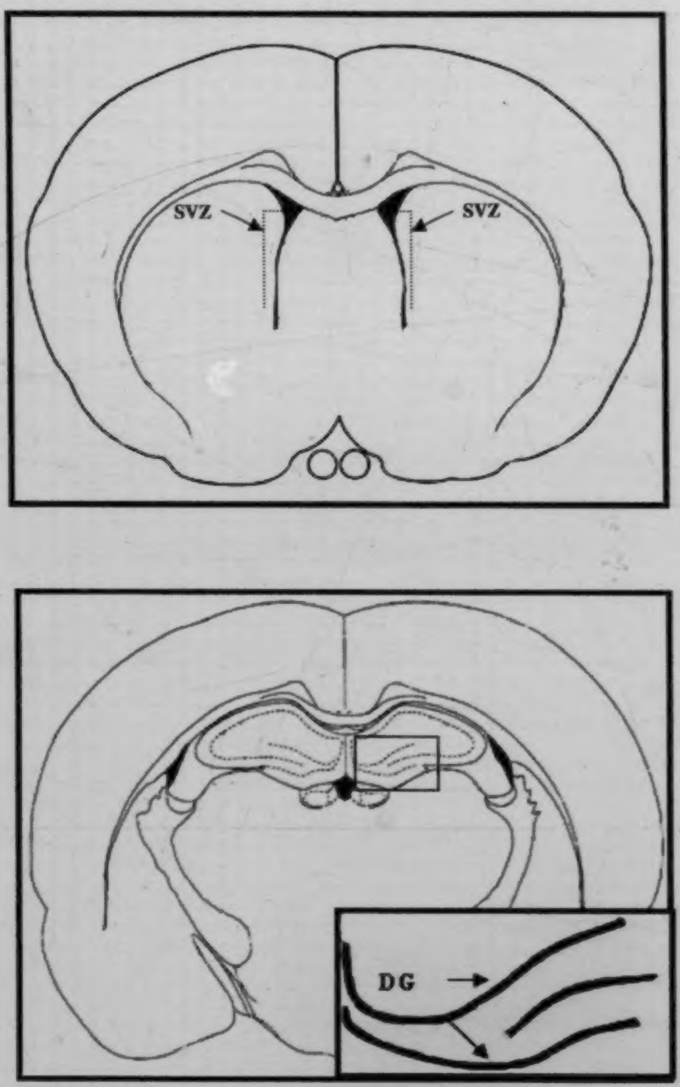


Figure 4.1. Subventricular Zone (SVZ) and Dentate Gyrus (DG) Reference Sections.

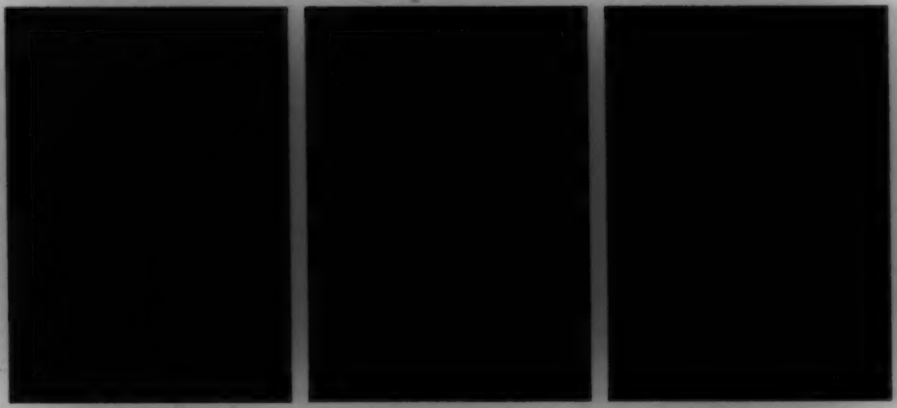


Figure 4.2. Immunostaining for 5-bromo-2-deoxyuridine (BrdU) and Ki-67 in the Subventricular Zone (SVZ). Representative photomicrographs illustrating the typical number of BrdU-positive cells in the SVZ of a naïve adult rat (A) and a post-natal-day-7 rat subject to hypoxia-ischemia (B). Immature animals have significantly more BrdU-positive cells in the SVZ. Immature animals also have numerous Ki-67 positive cells in the SVZ (C). Scale bar = 50μm.

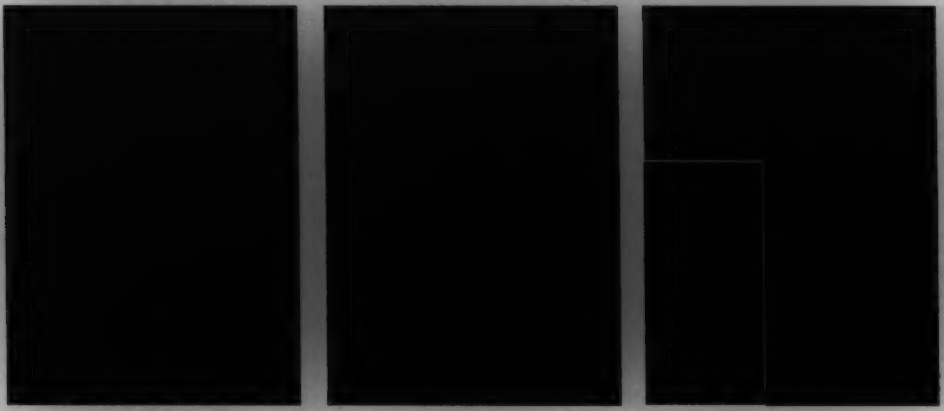
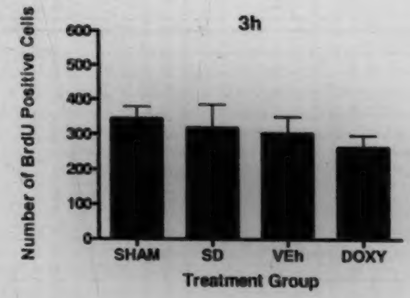
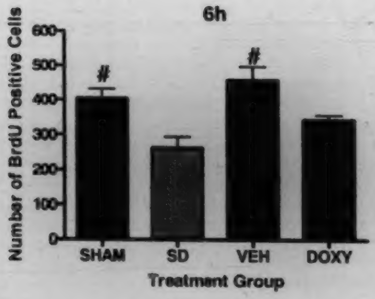
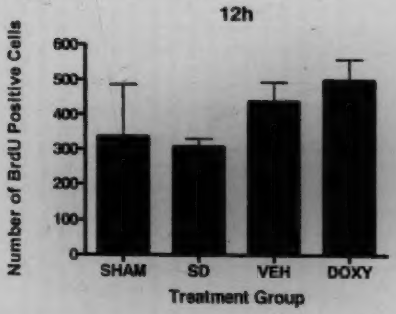
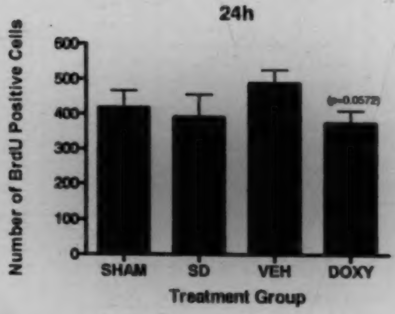


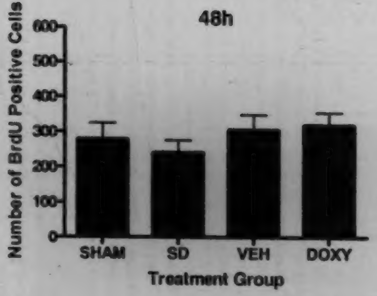
Figure 4.3. 5-bromo-2-deoxyuridine (BrdU) Immunostaining in the Subventricular Zone (SVZ). Representative photomicrographs illustrating the number, pattern, and distribution of BrdU-positive cells in the SVZ of rat pups, 24 hours after hypoxia-ischemia and treatment with saline vehicle (B) or doxycycline (DOXY,C). Inset in (C) shows significantly fewer BrdU-positive cells in DOXY-treated pups 6 hours post-HI. SHAM animal is shown in (A). Scale bar = 50μm.

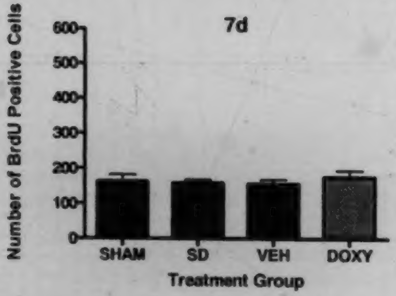

3h
Number of BrdU Positive Cells
0
100
200
300
400
500
600
SHAM
SD
VEh
DOXY
Treatment Group


6h
Number of BrdU Positive Cells
0
100
200
300
400
500
600
#
#
SHAM
SD
VEH
DOXY
Treatment Group


12h
Number of BrdU Positive Cells
0
100
200
300
400
500
600
SHAM
SD
VEH
DOXY
Treatment Group


24h
Number of BrdU Positive Cells
0
100
200
300
400
500
600
(p=0.0572)
SHAM
SD
VEH
DOXY
Treatment Group


48h
Number of BrdU Positive Cells
0
100
200
300
400
500
600
SHAM
SD
VEH
DOXY
Treatment Group


7d
Number of BrdU Positive Cells
0
100
200
300
400
500
600
SHAM
SD
VEH
DOXY
Treatment Group

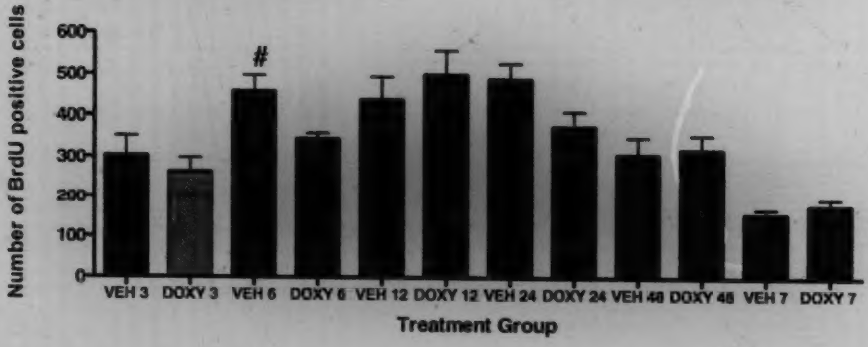
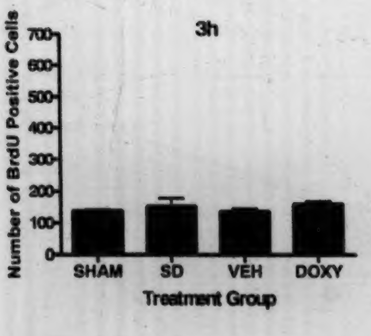
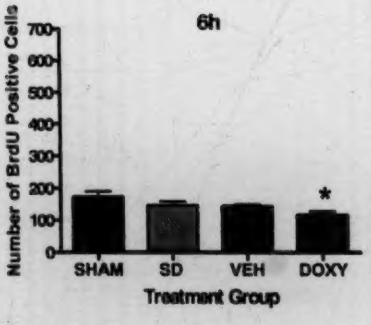
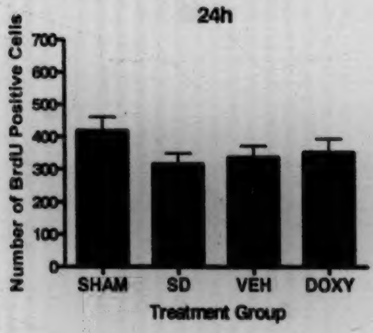

Number of BrdU positive cells
0
100
200
300
400
500
600
#
VEH 3
DOXY 3
VEH 6
DOXY 6
VEH 12
DOXY 12
VEH 24
DOXY 24
VEH 48
DOXY 48
VEH 7
DOXY 7
Treatment Group

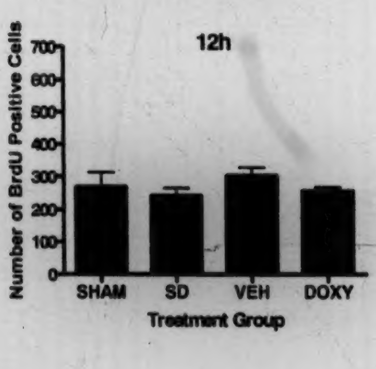
Figure 4.4. Number of 5-bromo-2-deoxyuridine (BrdU) Positive Cells in the Subventricular Zone (SVZ) 3 hours-7 days Post Hypoxia-Ischemia (HI). Histograms displaying the number of BrdU positive cells in the medial SVZ , 3h-7d post-HI, in SHAM, SHAM pups-treated with doxycycline (SD), vehicle-treated HI pups (VEH), and doxycycline-treated HI pups (DOXY). Treatment with DOXY significantly decreases the number of BrdU positive cells in both treated SHAM and HI animals, at 6 hours. Twenty-four hours post-HI, treatment with DOXY also decreases the number of BrdU positive cells, although this decrease is not significant. By 48h and 7 days post-HI there are no significant increases or decreases in any group. The compilation histogram depicts the relative increases and decreases in both DOXY and VEH-treated HI pups, throughout the timecourse investigated. It is noted that the number of BrdU positive cells increases 6-24h post HI and then tapers off 48h-7d post-HI. (# = significant difference from DOXY-treated pups). Values represent mean ± SD (n=6).

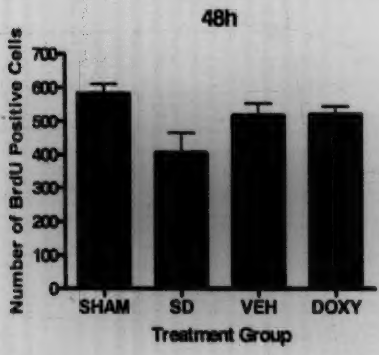
Figure 4.5. 5-bromo-2-deoxyuridine (BrdU) Immunostaining in the Dentate Gyrus (DG). Representative photomicrographs illustrating the number, pattern, and distribution of BrdU-positive cells in the DG of rat pups, 48 hours after hypoxia-ischemia and treatment with saline vehicle (B) or doxycycline (C). SHAM animal is shown in (A). Scale bar = 50μm.

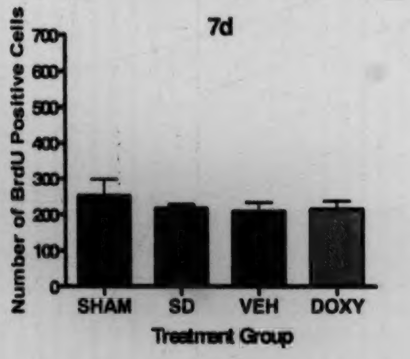

3h
Number of BrdU Positive Cells
0
100
200
300
400
500
600
700
SHAM
SD
VEH
DOXY
Treatment Group


6h
Number of BrdU Positive Cells
0
100
200
300
400
500
600
700
*
SHAM
SD
VEH
DOXY
Treatment Group


24h
Number of BrdU Positive Cells
0
100
200
300
400
500
600
700
SHAM
SD
VEH
DOXY
Treatment Group


12h
Number of BrdU Positive Cells
0
100
200
300
400
500
600
700
SHAM
SD
VEH
DOXY
Treatment Group


48h
Number of BrdU Positive Cells
0
100
200
300
400
500
600
700
SHAM
SD
VEH
DOXY
Treatment Group


7d
Number of BrdU Positive Cells
0
100
200
300
400
500
600
700
SHAM
SD
VEH
DOXY
Treatment Group

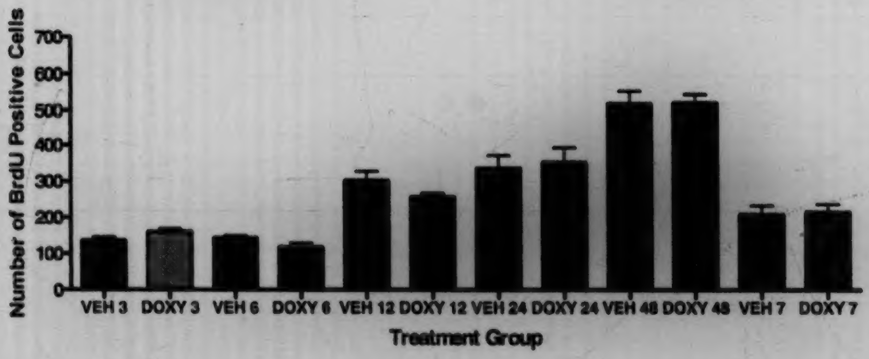

Number of BrdU Positive Cells
0
100
200
300
400
500
600
700
VEH 3
DOXY 3
VEH 6
DOXY 6
VEH 12
DOXY 12
VEH 24
DOXY 24
VEH 48
DOXY 48
VEH 7
DOXY 7
Treatment Group

Figure 4.6. Number of 5-bromo-2-deoxyuridine (BrdU) Positive Cells in the Dentate Gyrus (DG) 3 hours-7 days Post Hypoxia-Ischemia (HI). Histograms displaying the number of BrdU positive cells in the medial DG , 3h-7d post-HI, in SHAM, SHAM pups-treated with doxycycline (SD), vehicle-treated HI pups (VEH), and doxycycline-treated HI pups (DOXY). DOXY-treated HI pups have significantly fewer BrdU positive cells compared to SHAM animals at 6h. Twelve hours post-HI, treatment with DOXY also decreases the number of BrdU positive cells compared toVEH-treated pups, although this decrease is not significant. The compilation histogram depicts the relative increases and decreases in both DOXY and VEH-treated HI pups, throughout the timecourse investigated. It is noted that the number of BrdU positive cells peaks at 48h post HI and significantly tapers off 7d post-HI. (* = significant difference from SHAM pups). Values represent mean ± SD (n=6).

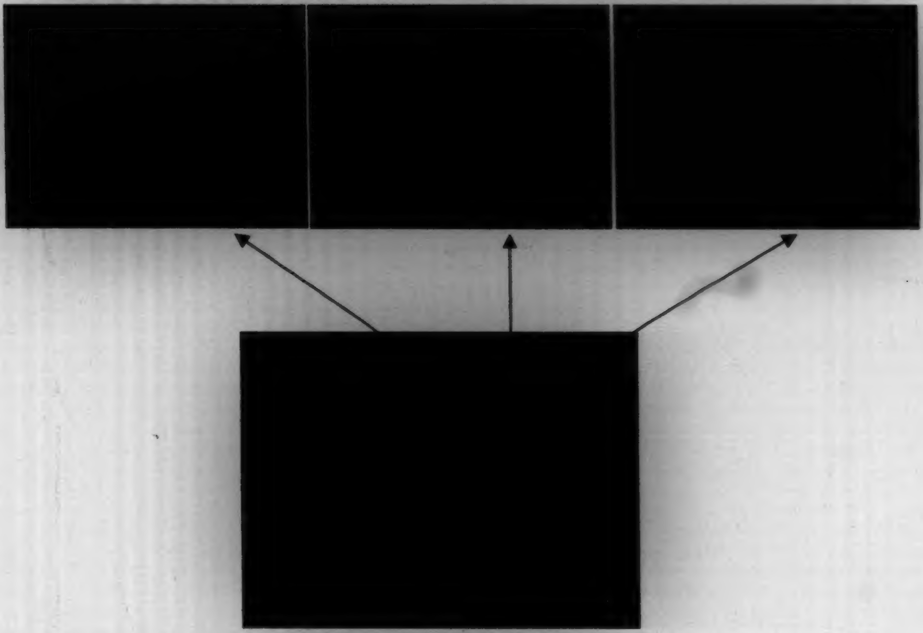


Figure 4.7. Double Immunostaining for Neuronal Nuclei (NeuN) and 5-bromo-2-deoxyuridine (BrdU). Representative photomicrographs depicting that the majority of BrdU- positive cells in the subventricular zone afer hypoxia-ischemia do not co-lococalize with the post-mitotic neuronal marker NeuN. A- BrdU positive Cells; B-NeuN-positive Cells; C-Overlay of BrdU and NeuN-positive cells showing lack of co-localization; D-Low maginification overlay image of the entire SVZ. Scale bar = 50µm in D; 10µm A-C.

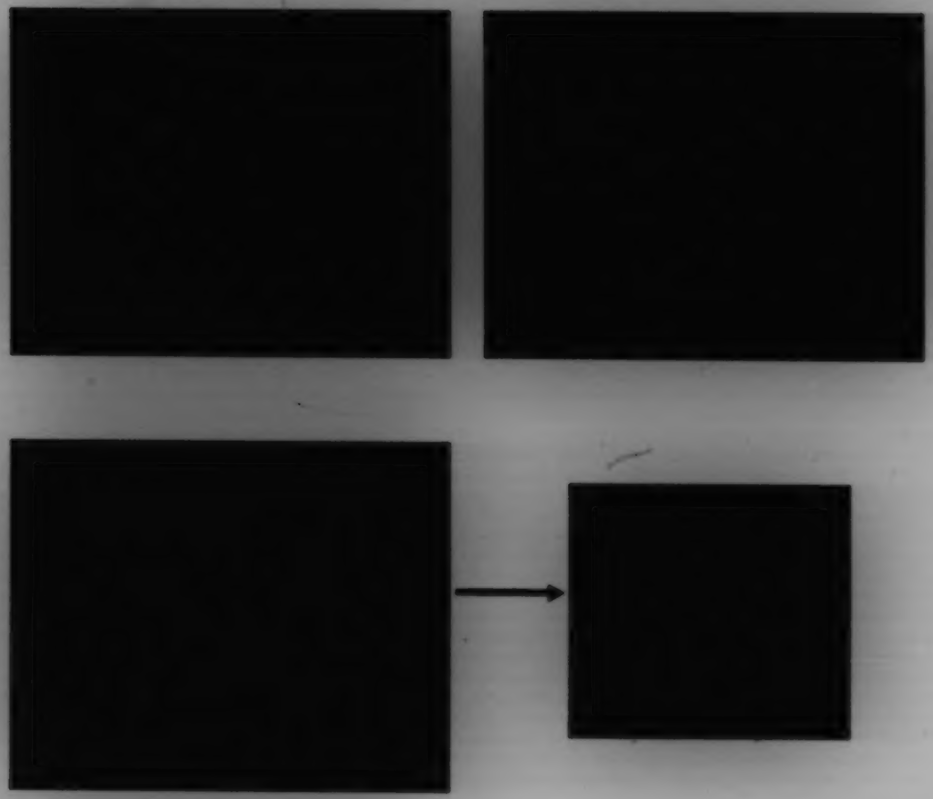


Figure 4.8. Double Immunostaining for 5-bromo-2-deoxyuridine (BrdU) and Glial Fibrillary Acidic Protein (GFAP). Double immunostaining for BrdU (A) and GFAP (B) depicting that the majority of BrdU-positive cells in hypoxic- ischemic rat pups do not co-localize with GFAP (C). Image from inset is shown in (D). Scale bar = 20μm.

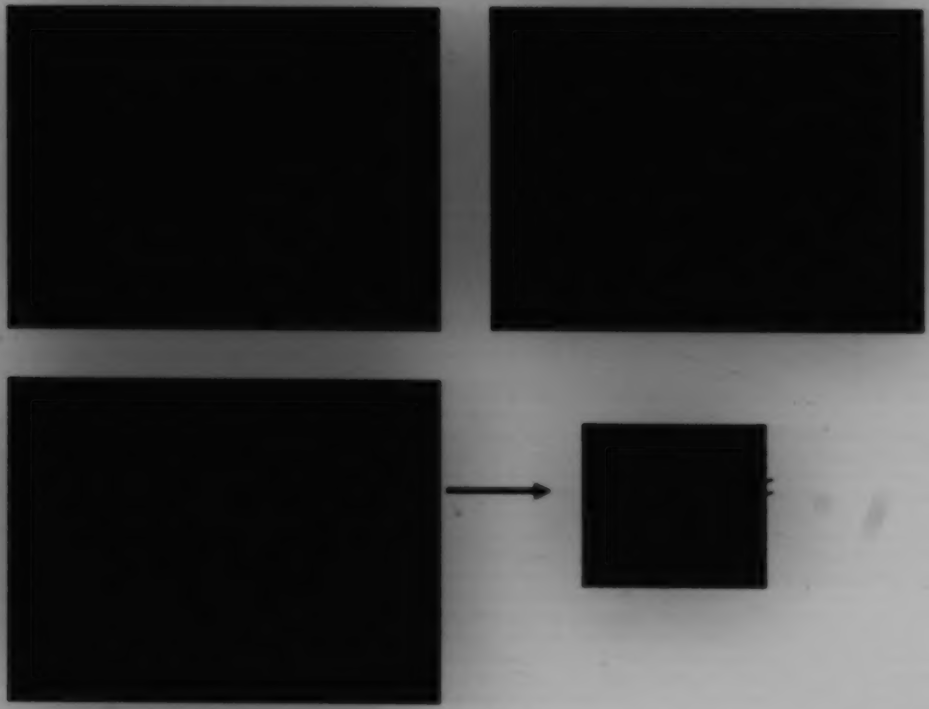


Figure 4.9. Double Immunostaining for 5-bromo-2-deoxyuridine (BrdU) and Cleaved Caspase-3. Representative photomicrographs illustrating that the majority of BrdU-positive cells in the subventricular zone, 12-hours post-HI, are negative for cleaved caspase-3, an effector of apoptotic cell death. A-BrdU positive cells; B-Cleaved Caspase-3 positive cells; C-Overlay image of A and B. D- Image from inset depicting that few BrdU/Cleaved Caspase-3 cells do exist on the border of the lateral ventricle. The co-localization in these cells appear yellow/orange. Scale bar = 20μm.

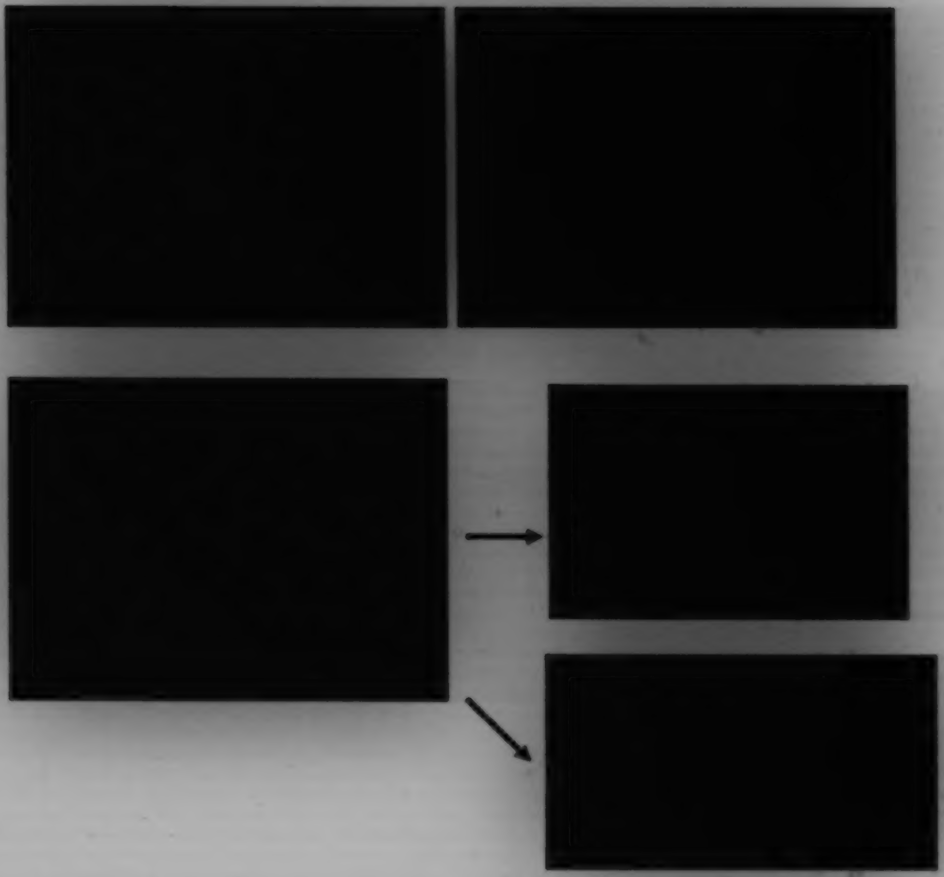


Figure 4.10. Double Immunostaining for 5-bromo-2-deoxyuridine (BrdU) and Cleaved Caspase-3. Representative photomicrographs depicting that more BrdU/cleaved caspase-3 positive cells (purple) exist in the subventricular zone (SVZ) 7d post-HI as compared to earlier time points. However, the majority of BrdU- positive cells in the SVZ do not co-localize with the end effector of apoptosis. A-BrdU positive cells in the SVZ; B-Cleaved Caspase-3 positive cells lining the lateral ventricle; C-Overlay image of A and B; D + E –Images from inset depicting that BrdU/Cleaved caspase-3 co-localizations appear as purple cells lining the lateral ventricle. Scale bar = 10μm.

Figure 4.11. Triple Immunostaining for Neurons (NeuN), Cleaved Caspase-3, and 5-bromo-2-deoxyuridine (BrdU) in the Subventricular Zone (SVZ) of Hypoxic-Ischemic (HI) Rat Pups. Representative photomicrographs illustrating that 7-days post HI, few BrdU positive cells co-localize with NeuN (yellow/orange). In addition, BrdU positive/cleaved caspase-3 positive cells are also visible (purple). Importantly, the BrdU positve/NeuN positive cells do not co-localize with cleaved caspase-3 indicating that these new cells of neuronal phenotype are negative for a cell death marker. Scale bar = 10μm.

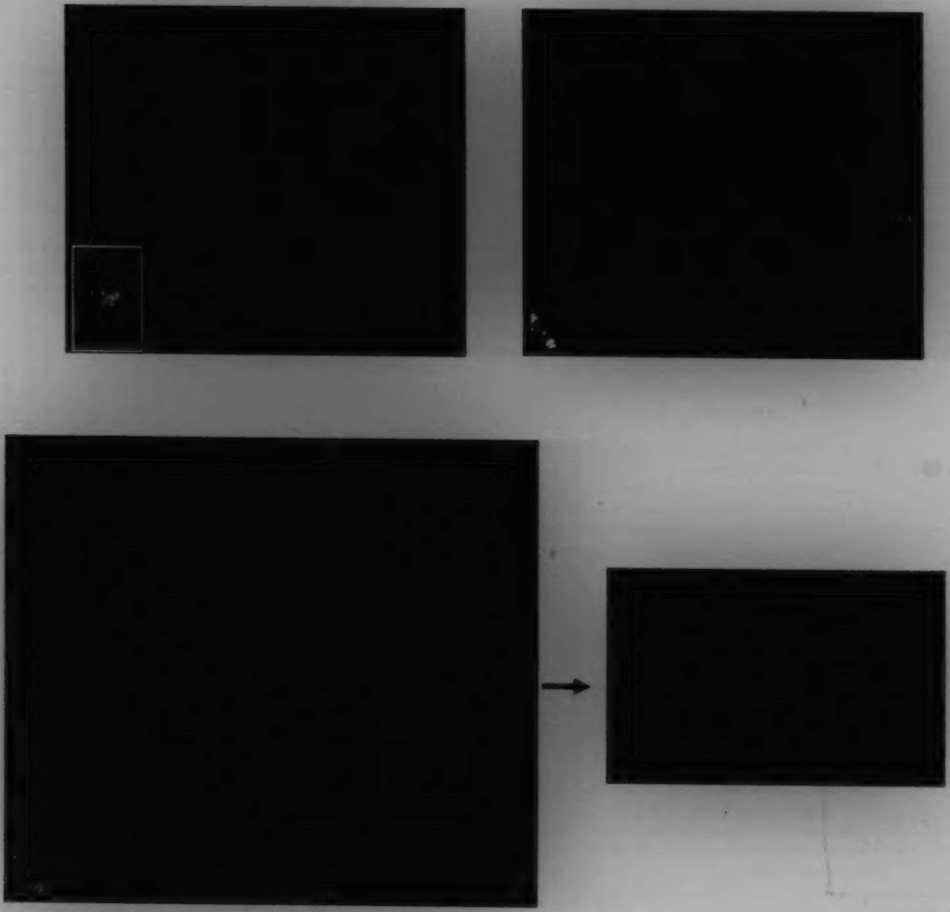


Figure 4.12. Double Immunostaining for Nestin and Glial Fibrillary Acidic Protein (GFAP) in the Subventricular Zone (SVZ) of Hypoxic-Ischemic Rat Pups. Representative photomicrographs indicating that the majority of nestin-positive cells (A) in the SVZ are negative for GFAP (B). Overlay of images A and B is shown in C. Notice the nestin/GFAP-positive cells (yellow/orange) shown medially in Figure C. High magnification image of these cells is shown in (D). Inset in (A) illustrates a nestin-positive neuron (green) at high magnification. Scale bar = 20μm.

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CHAPTER 5

Temporal and Regional Neurotrophin, Pro-Inflammatory, and Gelatinase Responses to Neonatal Hypoxia-Ischemia and Administration of Doxycycline

5.1. Introduction

Throughout this thesis, the dual nature of microglial activation has been highlighted. It is well established that microglia release deleterious factors upon activation in a pathological setting. Some of these factors include: superoxide anions, hydroxyl radicals, hydrogen peroxide, peroxynitrite, glutamate, quinolinic acid, histamine, eicosanoids, tumor necrosis factor α (TNFα) and interleukin-1β (IL-1β) (Nakajima & Kohsaka 2004, Lai & Todd 2006). However, activated microglia also release trophic factors that play crucial roles in development. Examples of trophic factors released by activated microglia include: brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), neurotrophin-3 (NT-3), neurotrophin-4/5 (NT-4/5), tumor growth factor β1 (TGFβ1), glial derived neurotrophic factor (GDNF), plasminogen, interleukin-6 (IL-6) and ciliary neurotrophic factor (CNTF) (Nakajima & Kohsaka 2004, Lai & Todd 2006). Through the release of these factors, microglia have important roles in the regulation of cell differentiation, cell number, synapse formation and axonal guidance. As such, the inhibition of microglia may have consequences after a pathological event that extends far beyond a reduction in neuroinflammation.

The toxic and trophic properties of microglia and subsequent activation have been well documented by other investigators. With respect to their specific protective effects, previous studies have demonstrated that the exogenous application of microglia provides protection against different types of ischemic injury and that microglia are involved in the attraction and differentiation of neural precursor cells

(Aarum *et al.* 2003, Lalancette-Hebert *et al.* 2007, Kitamura *et al.* 2004, Kitamura *et al.* 2005, Imai *et al.* 2007). Activated microglia are also an endogenous pool of neurotrophic molecules, such as BDNF, that provide trophic support for adjacent ischemic neurons (Lalancette-Hebert *et al.* 2007). After middle cerebral artery occlusion (MCAO), selective ablation of proliferating microglia is associated with 1) Marked deregulation of post-ischemic neuroinflammation; 2) Increased lesion size and 2.7 fold increases in the number of apoptotic neurons; and 3) Significant decreases in the levels of neurotrophins expressed by galectin-3 (Mac-2) positive-proliferating microglia in areas around the ischemic lesion (Lalancette-Hebert *et al.* 2007). BDNF is a neurotrophin with significant neuroprotective properties, and it too has been studied extensively in HI. Although the exact mechanism by which many neurotrophins exert their neuroprotective effects is not completely understood, BDNF has been shown to reduce the production of free radicals and to serve as an anti-apoptotic molecule through the blockade of caspase-3 activation and upregulation of Bcl-2 (Calvert & Zhang 2005, Han *et al.* 2000, Han & Holtzman 2000, Galvin & Oorschot 2003). Further, molecular mechanisms associated with the enhancement of cell genesis after HI may be related to BDNF, as it has also been documented to have important roles in cell proliferation in the SVZ (Iwai *et al.* 2006, Kokaia & Lindvall 2003).

On the other hand, microglial activation associated with inflammation has been shown to impair both basal and insult-induced hippocampal neurogenesis (Ekdahl *et al.* 2003). In this aspect, activated microglia have been found in close proximity to newly formed cells and it is believed that the impairment of neurogenesis depends on the degree of microglial activation and the deleterious effects mediated by pro-inflammatory cytokines such as IL-1β, and TNFα (Ekdahl *et al.* 2003). In a non-pathological state, the pro-inflammatory cytokines are vital for the differentiation of T- and B-lymphocytes and monocyte chemotaxis (Calvert & Zhang 2005). However, cytokines were originally implicated in the inflammatory response following HI because significant associations were observed between abnormal neurological outcome and cytokine concentration in umbilical cord blood taken from infants whose mothers had chorioamnionitis. Moreover, the highest concentrations of cytokines were found in infants who were diagnosed with HIE (Liu *et al.* 1994, Shalak *et al.* 2002, Silverstein *et al.* 1997).

Similar to the cytokines, matrix metalloproteinases (MMPs) have been implicated in both development and pathology of the nervous system (Yong 2005, Yong *et al.* 2001). MMPs are a family of zinc-dependent proteases that are classically described in the context of extracellular matrix remodeling, but more recent evidence suggests this family has important roles in the regulation of other functions such as cell-fate specification, neuronal precursor migration, myelinogeneis, angiogenesis, tumorgenesis, inflammation, neuronal death and cell signaling (Masuda *et al.* 2007, Kelly *et al.* 2006, Lee *et al.* 2006, Vaillant *et al.* 2003, Ohab & Carmichael 2007, Rosenberg 2002b). The activities of the metalloproteinases are tightly regulated, as these molecules are potent proteoloytic enzymes capable of widespread destruction and thus are largely absent in their active form in the normal CNS (Yong *et al.* 2001).

However, they do become upregulated after injury and in certain neurological disorders, such as stroke, multiple sclerosis and glioma, in response to growth factors, cytokines, chemokines, and extracellular matrix components (Rosenberg & Yang 2007, Bodey *et al.* 2000, Yong *et al.* 2007, Yong *et al.* 2001, Wild-Bode *et al.* 2001, Forsyth *et al.* 1999, Rosenberg 2002b, Rosenberg 2002a). Following activation, it has been documented that microglia can augment the immune response by releasing MMPs (Nuttall *et al.* 2007). Further, elevated expression of metalloproteinases in microglia may explain the high degree of microglial motility observed in CNS pathology (Nuttall *et al.* 2007). Although the roles of MMP-2 (gelatinase A, 72 kDa) and MMP-9 (gelatinase B, 92 kDa) have been well documented in relation to the breakdown of the blood-brain barrier after ischemia because of their substrate specificity for fibronectin, laminin, and collagen type IV (Heo *et al.* 1999, Kelly *et al.* 2006, Yong *et al.* 2007), to date no study has examined the temporal *and* regional profile of MMP activity after neonatal hypoxia-ischemia (Richards *et al.* 2006).

The previous chapter demonstrated that DOXY acutely affects the timecourse of cell genesis after hypoxic-ischemic brain injury. Even though there were no sustained changes in the number of BrdU-positive cells when evaluated 2 and 7d post-HI, questions still remain as to DOXY's exact mechanism of action in relation the survival of new cells, its neuroprotective properties in general, and the observed decrease in BrdU immunoreactivity 6h post-HI in the SVZ. Given the intimate connection between inflammation and development and the trophic and toxic factors released by microglia, the goal of this chapter was to provide further insight into the consequences of DOXY administration in neonatal HI by investigating changes in the major pro-inflammatory cytokines implicated in both neonatal development and pathology (TNFα, IL-1β), a neurotrophin that mediates neuroprotection (BDNF), and gelatinases that have a dual nature in development and repair (MMP-2 and -9).

5.2. Materials and Methods

5.2.1. Animals and Surgical Procedures

As described in detail in the earlier chapters, we used a well characterized procedure combining unilateral common carotid occlusion with systemic hypoxia to induce HI (Jantzie *et al.* 2005, Jantzie *et al.* 2006). The paradigm employed produces mild unilateral brain damage. As with all animal experiments performed, the procedures described are in accordance with the guidelines set forth by the Canadian Council on Animal Care and were approved by the Health Sciences Animal Policy and Welfare Committee of University of Alberta.

5.2.2. Drug Administration and Tissue Preparation

DOXY (SIGMA, 10 mg/kg) or saline vehicle (VEH) was administered intraperitoneally in a volume of 100 μl as a one-time dose immediately before HI. Pups were then euthanized by decapitation 3h, 6h, 12h, 24h, 48h or 7days after the HI insult (n=8), and their brains were removed and rapidly dissected in to frontal cortex, striatum and hippocampus. At the time of assay, these samples were removed from storage at -80°C. Regions were then homogenized in 100μl ddH_2O and this homogenate was aliquoted for both ELISA and Gelatin Zymography assays. Protein amounts in the homogenate were assessed using a bicinchoninic acid (BCA) protein assay (Sigma), in which bovine serum albumin (BSA) was used as a standard. Neurotrophin, proinflammatory cytokine and gelatinase activity were normalized to the protein amounts as determined by the assays.

5.2.3. Enzyme-Linked Immunosorbent Assays (ELISA)

DuoSet ELISA kits for IL-1β, TNF-α and BDNF were purchased from R&D Systems. These assays were carried out according to the protocols provided by the manufacturers with slight modifications. Briefly, samples were run in triplicate on sterilized 96 well plates. To begin, plates were coated with the appropriate capture antibody (anti-BDNF; anti-TNFα ; anti-IL-1β; all 1/500) and were allowed to stand overnight, at room temperature. The following day after shaking off excess capture antibody and washing with PBS, wells were incubated with ELISA diluent (1% bovine serum albumin, BSA) for 1h. After further washing, samples and standards also made in ELISA diluent, were pipetted accordingly and allowed to incubate for 2h. The detection primary antibody (biotinylated anti-BDNF; anti-TNFα; anti-IL-1β, all 1/500 in ELISA diluent) was then applied for 1-2h. After appropriate washes and incubation with horseradish peroxidase (HRP) strep-avidin (1/500 in ELISA diluent), 100μl of commercial tetramethylbenzidine (TMB, BioSource) were added to each well and plates were placed on a shaker. Upon development of the chromagen, ELISA "stop" solution (1.8N sulfuric acid) was added to each well, and plates were read on a plate reader held at 450 nm.

5.2.4. Gelatin Zymography

Regional samples were homogenized in 100µl ddH_2O. A 30 µl aliquot was taken and added to 75 µl lysis buffer containing 50 mM Tris–HCl, 320 mM sucrose, 1 mM dithiothreitol, 10 µg/mL leupeptin, 10 µg/mL soybean trypsin inhibitor, and 2 µg/mL aprotinin. After centrifugation at 12000 rcf for 5min, supernatant was collected and combined with 20 µl gelatin sepharose in a tube which was rotated at 4°C for 1h. Following further centrifugation and the addition of binding and elution buffers, prepared samples were combined with loading dye according to protein amounts determined by prior protein analyses. Samples were loaded on a pre-cast Novex® 10% zymogram (gelatin) gels (Invitrogen) and run at 150V for 2.0-2.5h at 4°C. Purified human MMP standards (Oncogene) were loaded in one lane at a final protein concentration of 10 µg/100 µl. Upon separation by electrophoresis, gels were incubated in renaturing buffer (Triton-X100 2.5% v/v) for 1 h. Gels were then incubated in incubating buffer solution (50 mM Tris–HCl, 0.15 M NaCl, 5 mM $CaCl_2$) at 37.5°C for 4d. Gels were stained with Coomassie blue R-250 for 1 h and destained accordingly. MMP activation appeared as transparent bands on a blue background. Images of gels were captured by digital photographs taken on a MCID image analysis system; bands were quantified by densitometry using Adobe Photoshop Elements. Images of representative gels were reversed for display in the Figures section of this chapter.

5.2.5. Statistical Analysis

All results shown are expressed as mean ± standard deviation. Data were analyzed by one-way ANOVA followed by the Newman-Keuls multiple comparison test on significant main effects and interactions. Where appropriate, unpaired t-tests were used and when variances between two groups were significantly different a Welch's correction was employed. The general convention of a probability value of $p<0.05$ was used to establish statistical significance.

5.3. Results

5.3.1. Three Hours Post-HI: Levels of IL-1β, TNFα & BDNF and Gelatinase Activity.

Three hours post-HI, VEH-treated pups had significantly increased IL-1β in the hippocampus ipsilateral to common carotid artery ligation. Further, treatment with DOXY attenuated this increase (Figure 5.1). In the frontal cortex and striatum, BDNF was significantly decreased in both VEH and DOXY-treated pups compared to SHAM (Figure 5.1). Significant changes in gelatinase activity were evidenced by regional differences in MMP-9 and MMP-2 following HI. As shown in Figure 5.2, DOXY-treated pups significantly increased MMP-9 activity in the frontal cortex compared to SHAM animals. In some animals, treatment with DOXY attenuated the activities of both MMP-9 and MMP-2 in the frontal cortex and hippocampus (zymogram, Figure 5.2, $p > 0.05$).

5.3.2. Six Hours Post-HI: Levels of IL-1β, TNFα & BDNF and Gelatinase Activity.

HI caused significant increases in IL-1β in the striatum and TNFα in the frontal cortex and hippocampus, 6h post-HI (Figure 5.3). The administration of DOXY normalized the levels of these pro-inflammatory cytokines, as DOXY-treated pups had significantly lower levels of IL-1β and TNFα compared to VEH-treated pups (Figure 5.3). Pups treated with DOXY also had significantly lower levels of TNFα in the striatum. In the hippocampus, all HI pups had significantly elevated levels of BDNF and administration of DOXY significantly increased BDNF compared to VEH-treated pups.

Significant increases in the activity of MMP-9 were observed in the striatum and hippocampus, 6h post-HI (Figure 5.4). In contrast to the trend observed in the frontal cortex where DOXY-treated pups tended to have reduced MMP activity (see zymogram), the administration of DOXY did not affect the activity of MMP-9 in neither the hippocampus nor striatum, 6 hours post-HI.

5.3.3. Twelve Hours Post-HI: Levels of IL-1β, TNFα & BDNF and Gelatinase Activity.

A regional difference in IL-1β, TNFα and BDNF expression was clearly demonstrated 12h following HI. In the frontal cortex, DOXY-treated pups were found to have significantly elevated levels of IL-1β and TNFα compared to SHAM pups. In addition, those pups treated with DOXY also had significantly increased IL-1β compared to VEH-treated pups. In the striatum however, DOXY-treated pups had increased TNFα and BDNF compared to both VEH-treated and SHAM pups (Figure 5.5). All HI pups exhibited significant elevations in IL-1β in the hippocampus.

As displayed in Figure 5.6, the activity of MMP-9 was upregulated in the frontal cortex of both DOXY- and VEH-treated pups, 12h post-HI. Compared to SHAM, pro-MMP-2 activity was also significantly elevated. In the striatum, MMP-9 and the pro-form of MMP-2 showed significantly increased activity in VEH-treated pups only. There were no significant changes in MMP activity observed in the hippocampus, 12h post-HI.

5.3.4. Twenty-Four Hours Post-HI: Levels of IL-1β, TNFα & BDNF and Gelatinase Activity.

IL-1β was significantly increased in the hippocampus of both VEH and DOXY-treated pups, 24h post-HI (Figure 5.7). In this region, VEH-treated pups also had significantly increased BDNF. Although treatment with DOXY decreased BDNF to a level comparable to that observed in SHAMs, this reduction was not statistically significant (p=0.0581). Unlike the hippocampus, BDNF was significantly increased in the striatum of DOXY-treated pups in relation to both VEH-treated HI pups and SHAM operated controls.

No significant increases or decreases in gelatinase activity were observed in any region 24h post-HI. However as evidenced by the representative zymogram and histograms in Figure 5.8, treatment with DOXY tended to reduce the activities of both MMP-9 and MMP-2 in the frontal cortex at this timepoint.

5.3.5. Forty-Eight Hours Post-HI: Levels of IL-1β, TNFα & BDNF and Gelatinase Activity.

By 48h post-HI, the levels of BDNF and the pro-inflammatory cytokines stabilized in all regions as there were no significant changes recorded between groups (Figure 5.9). However, MMP-9 showed significantly increased activity in the hippocampus of DOXY-and VEH-treated pups, compared to SHAM (Figure 5.10). In addition, treatment with DOXY significantly increased the activity of MMP-2 compared to VEH-treated pups.

5.3.6. Seven Days Post-HI: Levels of IL-1β, TNFα & BDNF and Gelatinase Activity.

Although stabilization was observed in the levels of IL-1β, TNFα and BDNF 48h post-HI, as time passed after the injury, seemingly chronic changes in these variables were identified. Beginning in the frontal cortex, HI pups had significantly elevated levels of IL-1β and BDNF (Figure 5.11). Treatment with DOXY resulted in a significant increase in IL-1β compared to the levels observed in pups treated with saline VEH. In the striatum a distinct and consistent pattern immerged as VEH-treated pups had significantly increased IL-1β, TNFα and BDNF compared to SHAM. Treatment with DOXY reliably and significantly reduced the levels of BDNF and TNFα. The administration of DOXY also decreased and normalized the levels of IL-

1β in the striatum, although this reduction just failed to meet statistical significance (p=0.051). In the hippocampus, both DOXY and VEH-treated pups had significantly elevated levels of IL-1β 7d post-HI.

As depicted in the histograms and zymogram in Figure 5.12, changes in the activity of the gelatinases 7d post-HI were restricted to the striatum and hippocampus. Increased MMP-9 and MMP-2 activity was present in the hippocampus of both DOXY and VEH-treated pups. In addition, activity of pro-MMP-9 was increased the striatum of VEH-treated pups, 7d post-HI.

5.4. Discussion

The results of this investigation demonstrate the regional and temporal evolution of the pro-inflammatory cytokines IL-1β and TNFα, the neurotrophin BDNF, and gelatinolytic activity after neonatal HI. To our knowledge, this is the first report to suggest that responses to doxycycline administration differ by region following HI. In this study, P7 rat pups were pre-treated with DOXY or saline VEH and then allowed to survive on a timecourse ranging from 3h to 7d post-HI. In the first 3 hours following HI, it was found that HI caused acute reductions in BDNF in the frontal cortex and striatum ipsilateral to carotid artery ligation. In the hippocampus, HI significantly increased IL-1β, but this increase was significantly attenuated in DOXY-treated pups. Accompanying these findings, noticeable elevations in MMP-9 activity were documented in the frontal cortex. Although there was a trend for MMP-9 and MMP-2 activity to be reduced in DOXY-treated pups, the decreases observed failed to reach statistical significance. These results are important because they provide crucial information as to how different regions vulnerable to HI, respond acutely after injury. It has been previously reported that MMP-9 is upregulated 1-3d after stroke, within central ischemic areas (Zhao *et al.* 2006). In contrast to MMP-2 which is constitutively expressed in the brain, MMP-9 activity is induced in pathological situations. It was clear that only MMP-9 activity was induced acutely in the frontal cortex. These results also show DOXY significantly attenuates IL-1β increases after HI, and these findings are in concert with previous *in vitro* reports that DOXY is capable of modulating neuroinflammation after hypoxia (Lai & Todd 2006).

In contrast to the regional patterns observed 3h after HI, different trends were observed 6h after HI injury. The role of TNFα after HI was also elucidated and coinciding with the previous data indicating that MMP-9 is upregulated after acute cerebral injury, significant increases in the activity of both forms of MMP-9 were documented in the striatum and hippocampus 6h post-HI. Unlike what was noted 3h post-HI, TNFα was significantly elevated in VEH-treated pups in both the frontal cortex and hippocampus. Treatment with DOXY significantly decreased levels of TNFα compared to the VEH-treated pups in every region examined, including the striatum. These data highlight the temporal role of the pro-inflammatory cytokines in the evolution and propagation of neuroinflammation after HI, with IL-1β being upregulated in response to injury first at 3h, followed by increases in TNFα at 6h. Further, DOXY's ability to inhibit neuroinflammation in a significant, temporal- and regional-dependent manner is clearly demonstrated at this point along the examined timecourse.

In the hippocampus 6h post-HI, BDNF increased in response to HI and most interestingly, treatment with DOXY further increased BDNF to levels that were significantly different from the VEH-treated pups. Although the mechanism of this is presently unknown, BDNF has been documented to be neuroprotective after neonatal HI and it is known to activate protective cascades in neurons and glia (Han *et al.* 2000, Cheng *et al.* 1997, Almli *et al.* 2000, Han & Holtzman 2000). As microglia are not the sole source of BDNF in the brain, this DOXY-dependent increase in BDNF may be a result of the augmentation of many cell types and may suggest that DOXY's ability to inhibit microglia does not affect their protective capacity. These data are consistent with previous studies on the effects of tetracyclines on microglia *in vitro*. Specifically, it has been shown that DOXY offers neuroprotection via the overall down-regulation of microglial factors, but that hypoxic stimulation of BDNF and GDNF is not negatively affected by its application (Lai & Todd 2006). The data presented in the current investigation also indicate that a portion of microglial protective machinery continues to function even in the presence of tetracyclines, as evidenced by the aforementioned finding in the hippocampus and the data showing that DOXY dependently increases BDNF up to 12h post-HI in the striatum.

Twelve hours post HI, the pattern of IL-1β, TNFα and BDNF changed dramatically from observations at earlier time points, and with the exception of IL-1β in the hippocampus, there were few changes related to HI alone. Rather, DOXY-treated pups had increased IL-1β in the frontal cortex and increased TNFα and BDNF in the striatum. Accompanying these findings, increased activity of MMP-9 and MMP-2 was documented in all HI pups in the frontal cortex, but only in VEH-treated pups in the striatum. The emergence of this pattern suggests that depending on the time and region after HI injury, DOXY is able to inhibit or augment the release of proinflammatory cytokines and potentially the activity of MMPs. Twenty-fours hours after HI, no significant changes in MMP activity were documented; however the trend for DOXY to decrease MMP-9 activity continued in the frontal cortex. The apparent decrease in MMP-9 activity in DOXY-treated pups is clearly observable when looking at gelatin zymograms, but statistical significance does not exist when luminosity analyses are performed. A similar trend existed in terms of IL-1β, TNFα and BDNF levels observed. DOXY decreased levels of all three of these factors as compared to VEH-treated pups, and although these reductions normalized these parameters to the levels observed in SHAM pups, they failed to reach statistical significance. However, in the striatum, DOXY-treated pups maintained increased BDNF levels compared to the other groups. These observations may represent a shift from acute responses to the pathological event, to recovery from injury and increased cellular efforts to maintain homeostasis. This notion is strengthened by the finding that by 48h post-HI there were no significant changes in IL-1β, TNFα and BDNF in any region examined.

In contrast to the stabilization observed two days after HI, evidence of seemingly chronic changes in the cytokines, BDNF and MMPs were obvious after 7 days. In the striatum, a distinct pattern emerged such that IL-1β, TNFα and BDNF were each significantly increased in VEH-treated pups compared to SHAM animals. When differences between treatment groups were analyzed, it was discovered that the administration of DOXY significantly attenuated the increases in TNFα and BDNF. This pattern was also observed with IL-1β, although the reduction caused by DOXY just failed to reach significance (p=0.051). This is the first time that a single dose of DOXY given prior to a HI has been shown to persistently reduce pro-inflammatory cytokine and BDNF levels a week beyond the initial injury. The consequences of these changes could be numerous. Overall, they could reflect a beneficial reduction in neuroinflammation initiated by DOXY. It is speculated that the reductions caused by DOXY are beneficial, as only VEH-treated pups had significantly increased levels of IL-1β, TNFα and BDNF compared to SHAMs. In this respect, administration of DOXY, at least in the striatum, seemed to maintain homeostasis. The increases in IL-1β documented in both VEH- and DOXY-treated pups in the frontal cortex and hippocampus may reflect persistent upregulation of the major proteins involved in neuroinflammation post-HI that could not be overcome with a single dose of DOXY, but may also be the beginning of a beneficial reparative response and an upregulation related to recovery after HI.

Tetracycline antibiotics are pluripotent drugs that act through direct and indirect mechanisms to inhibit neuroinflammation, MMP activity and affect many cellular functions, including proliferation, migration, and vascular remodeling (Lee *et al.* 2004, Burggraf *et al.* 2007). Although we have documented DOXY's ability to inhibit microglial activation and decrease neuroinflammation, our compiled data did not show significant DOXY-dependent MMP inhibition along the tested timecourse. Although this finding was counterintuitive, large intragroup variation in MMP activity was observed and is the most probable explanation for this finding, especially when the strong trends toward DOXY-inhibition under the most acute survival settings (3-6h) are considered. Although this would likely be rectified by increasing the n's in this portion of the study (n=3/group), other investigators have documented variability in MMP inhibition by tetracyclines and suggest that singular administrations of these drugs may not be enough to inhibit MMP activity (Machado *et al.* 2006). Further, MMP inhibition by DOXY may be mediated through many biochemical pathways and may be heavily dependent on pharmacokinetics. Besides small sample size, it is plausible that the variation observed could be related to inconsistent levels of DOXY in the rat pup brain. Reports on plasma and cerebral spinal fluid (CSF) levels of minocycline have indicated that there can be great variability after intraperitoneal injections (Fagan *et al.* 2004). Even though we quantified parenchymal DOXY levels in Chapter 2, it is important to note that intragroup variation was also observed in our studies. Regardless, when the data from this chapter are combined with the previous three, we detail that the administration of DOXY in HI injury significantly neuroprotects, and reduces microglial activation, cleaved caspase-3 and pro-inflammatory cytokines concurrent with non-statistically significant changes in MMP activity. Moreover, like other studies showing that MMP inhibition occurs at doses lower than those required for antimicrobial effects, we show that inhibition of neuroinflammation occurs at a dosage lower than that required for MMP activity inhibition. To rephrase, we show that the threshold required for DOXY's anti-inflammatory effects is lower than the threshold required for MMP inhibition. Also, in the first 3-6h after HI, one dose of DOXY is sufficient to inhibit the MMP activity in 2 out of 3 animals studied. Further, the roles of MMPs in pathology notwithstanding, these enzymes are crucial to neuronal plasticity and angiogenesis (Zhao *et al.* 2006). In light of data that suggest MMPs can contribute to cerebral remodeling by modulating the extracellular matrix, facilitating dendritic plasticity and by degrading inhibitory matrix or glial scars to allow axonal extension and recovery (Zhao *et al.* 2006, Yong *et al.* 2007, Agrawal *et al.* 2008), chronic inhibition of MMPs would likely be detrimental to recovery post-HI and should hinder subsequent neurodevelopment.

The particular factors we examined in this study represent only a small portion of the large inventory of microglial effectors. Perhaps the findings of this study are most interesting when combined with the data gathered in the previous chapter indicating that there were no persistent changes in BrdU immunoreactivity post-HI in DOXY-treated pups. As mentioned previously, MMP-9 is believed to be most responsive to acute brain injury (Wang *et al.* 2000, Wang *et al.* 2003, Lee *et al.* 2006). Nevertheless, MMP inhibition suppresses the neurogenic migratory response induced by stroke, and it is believed that MMPs should facilitate the endogenous recovery response in an injured brain (Lee *et al.* 2006). In light of the fact that we did not see any persistent inhibition of MMP activity following HI, it is plausible that MMP-dependent repair could continue as normal and that MMP-dependent neurogenenic migration would be unaffected by the administration of DOXY. As will be discussed in greater detail in the General discussion, it may be beneficial to extend these studies further to investigate neurogenesis and MMP activity over a longer timecourse after HI injury with repeated administrations of DOXY.

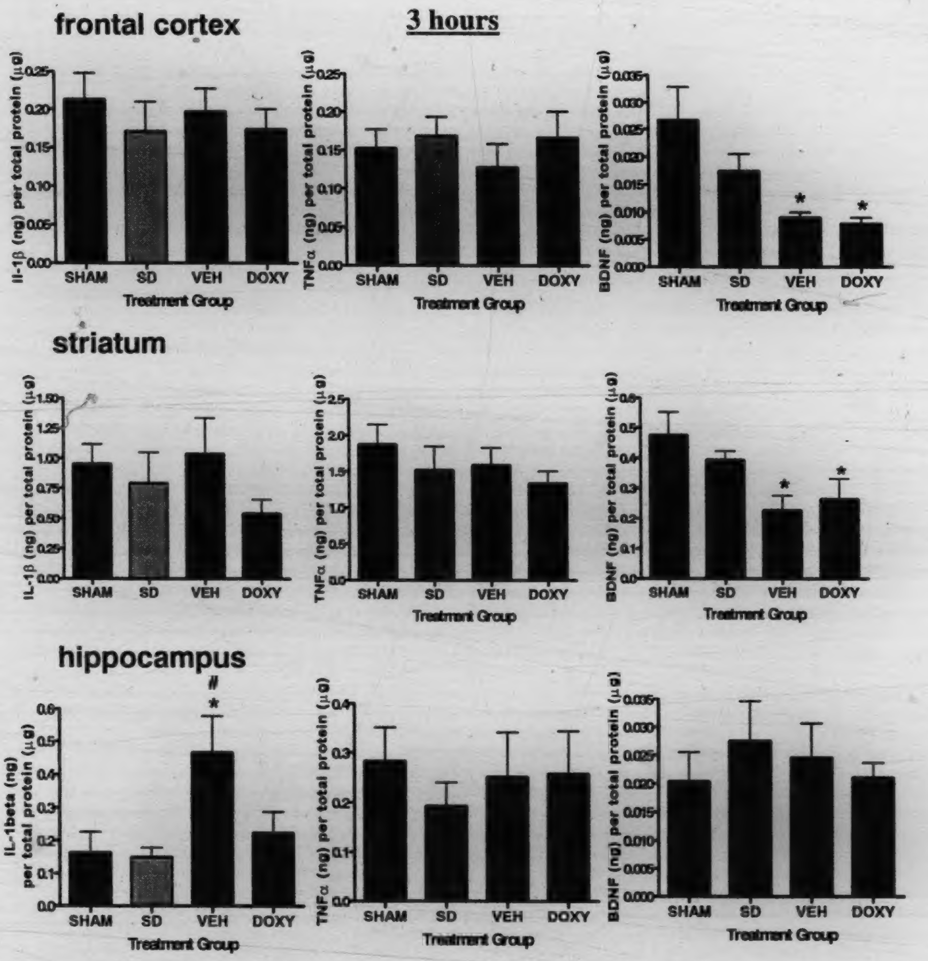
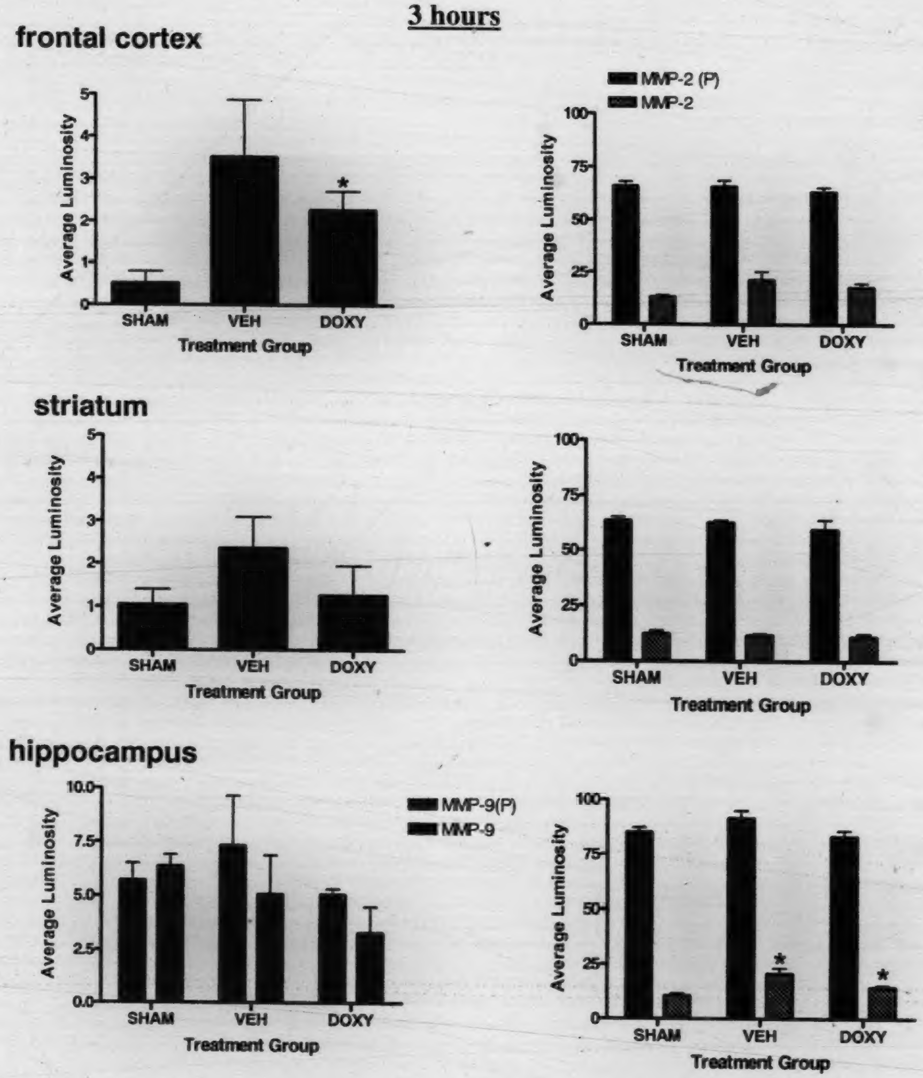
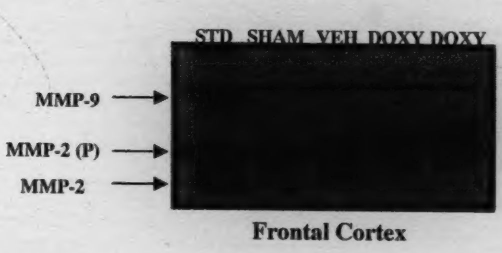


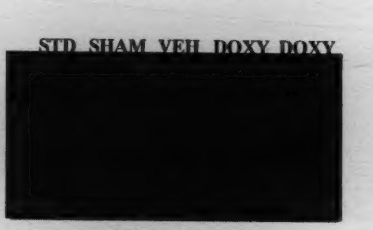
Figure 5.1. Levels of Interleukin-1β (IL-1β), Tumor Necrosis Factor α (TNFα) and Brain Derived Neurotrophic Factor (BDNF), in the Frontal Cortex, Striatum and Hippocampus Ipsilateral to Common Carotid Artery Ligation, 3 hours Post Hypoxia-Ischemia (HI). Neonatal HI, independent of treatment with doxycycline (DOXY) or saline vehicle (VEH), causes significant reductions in BDNF in both the frontal cortex and striatum as compared to the levels observed in SHAM pups. VEH-treated pups have significantly elevated IL-1β in the hippocampus 3 hours post-HI. DOXY-treated pups have significantly lower levels of IL-1β compared to VEH-treated pups, in the hippocampus. (* = significantly different from SHAM, # = significantly different from DOXY-treated HI pups). Values represent mean ± SD (n=8).

3 hours




STD SHAM VEH DOXY DOXY
MMP-9
MMP-2 (P)
MMP-2

Frontal Cortex


STD SHAM VEH DOXY DOXY

Hippocampus

Figure 5.2. Gelatinase Activity in the Frontal Cortex, Striatum and Hippocampus Ipsilateral to Carotid Ligation, 3 hours post Hypoxia-Ischemia (HI). Representative histograms and zymograms demonstrating that HI pups treated with doxycycline (DOXY) have significantly increased MMP-9 activity compared to SHAM pups in the frontal cortex, 3h post-HI. Both vehicle- (VEH) and DOXY-treated pups have significantly increased MMP-2 in the hippocampus. Treatment with DOXY tended to decrease the activities of both forms of MMP-9 and active MMP-2 in the cortex and hippocampus, although these decreases failed to reach significance. (* = significantly different from SHAM. # = significantly different from DOXY-treated HI pups). Values represent mean luminosity ± SD (n=3).

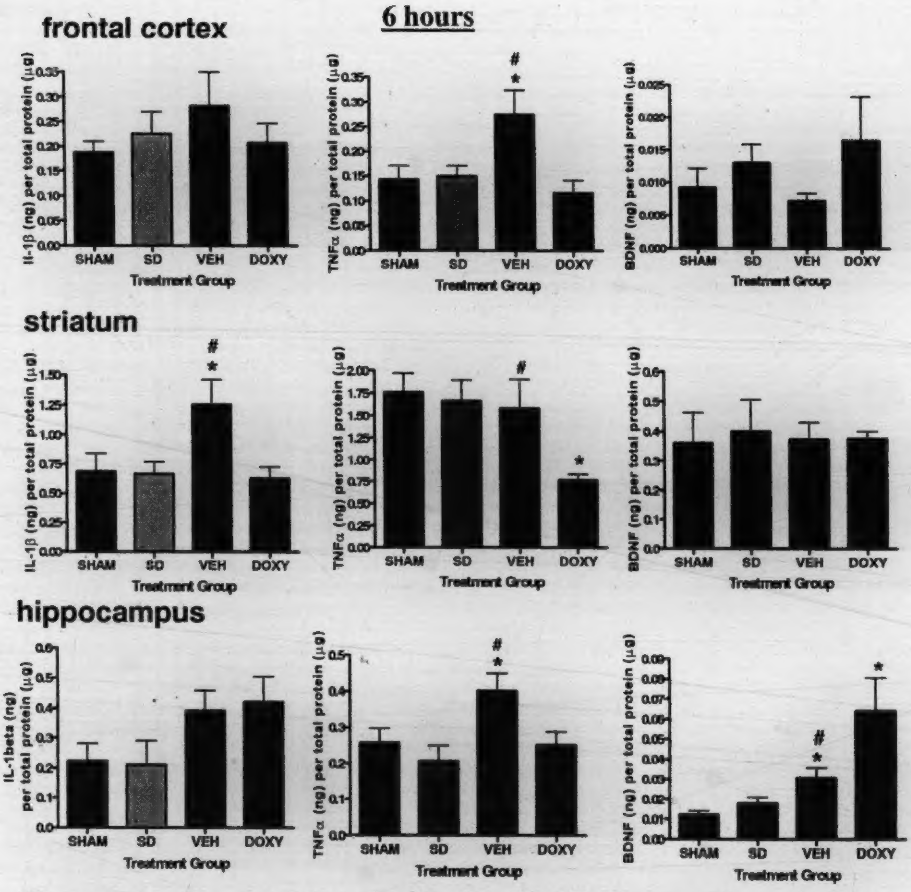
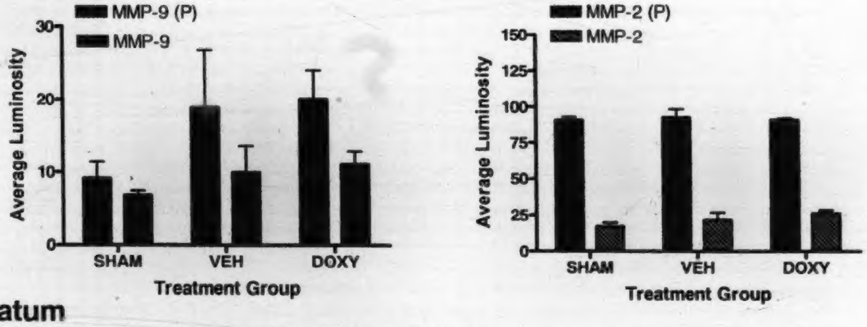


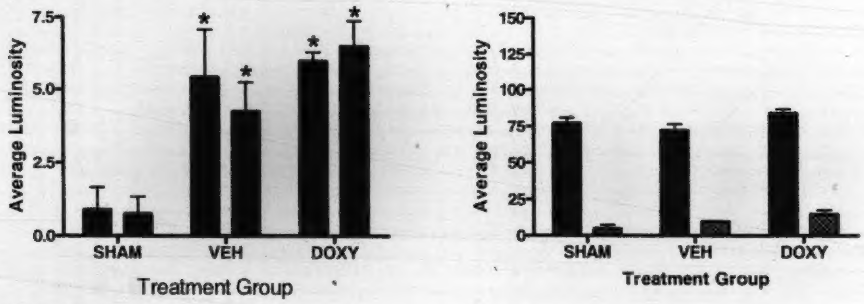
Figure 5.3. Levels of Interleukin-1β (IL-1β), Tumor Necrosis Factor α (TNFα) and Brain Derived Neurotrophic Factor (BDNF), in the Frontal Cortex, Striatum and Hippocampus Ipsilateral to Common Carotid Artery Ligation, 6 hours post Hypoxia-Ischemia (HI). IL-1β is significantly increased in the striatum of VEH-treated pups, 6 hours post-HI. DOXY-treated pups have significantly less IL-1β in the striatum. DOXY- treated pups also have significantly lower levels of TNFα in the striatum, 6 hours post-HI compared to VEH-treated pups. Treatment with DOXY also normalizes TNFα in the frontal cortex and hippocampus. BDNF levels are significantly increased in HI pups in the hippocampus, those pups treated with DOXY have significantly increased BDNF compared to VEH-treated pups. (* = significantly different from SHAM, # = significantly different from DOXY-treated HI pups). Values represent mean ± SD (n=8).

6 hours

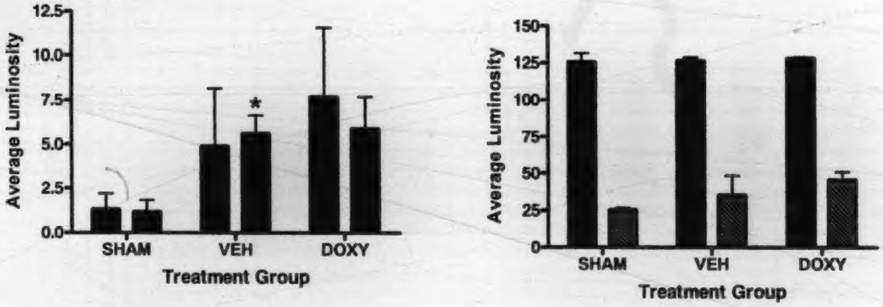
frontal cortex

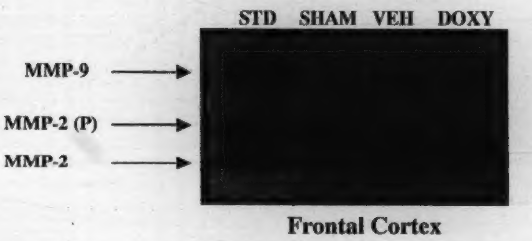


striatum



hippocampus




STD
SHAM
VEH
DOXY
MMP-9
MMP-2 (P)
MMP-2

Frontal Cortex

STD
SHAM
DOXY
VEH

Striatum

Figure 5.4. Gelatinase Activity in the Frontal Cortex, Striatum and Hippocampus Ipsilateral to Carotid Ligation, 6 hours Post Hypoxia-Ischemia (HI). Representative histograms and zymograms demonstrating that HI pups treated with either doxycycline (DOXY) and vehicle (VEH) have significantly increased MMP-9 activity compared to SHAM pups in the striatum, 6h post-HI. This increase in gelatinase activity was observed in both forms of MMP-9. In the hippocampus, VEH- treated pups exhibited significantly increased MMP-9. (* = significantly different from SHAM. # = significantly different from DOXY-treated HI pups). Values represent mean luminosity ± SD (n=3).

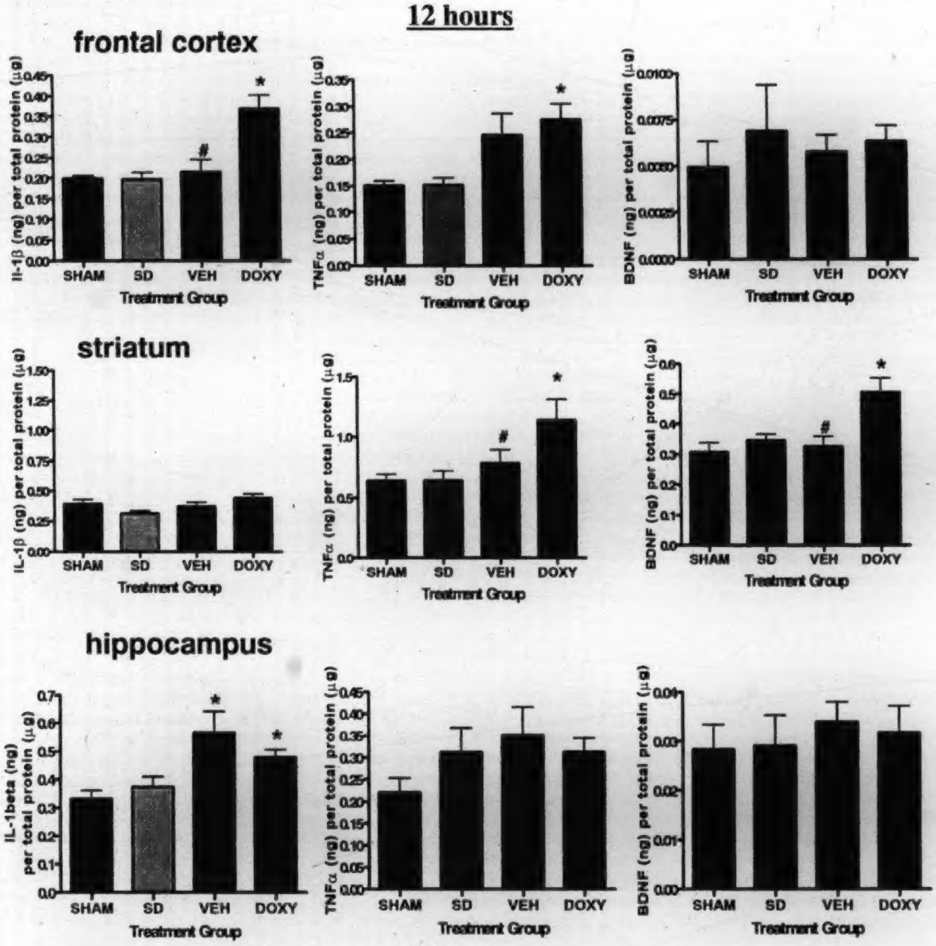
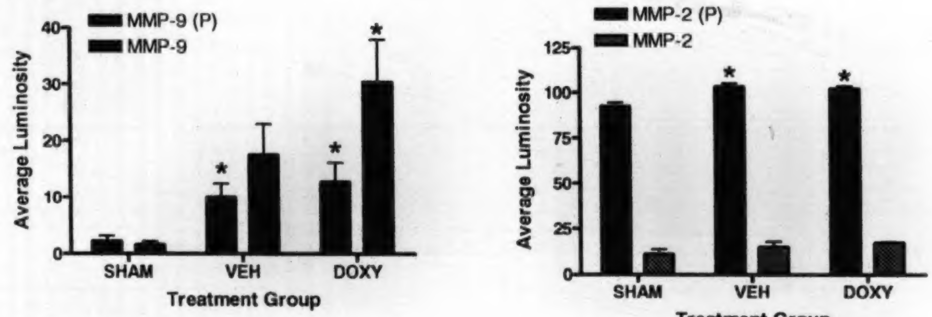


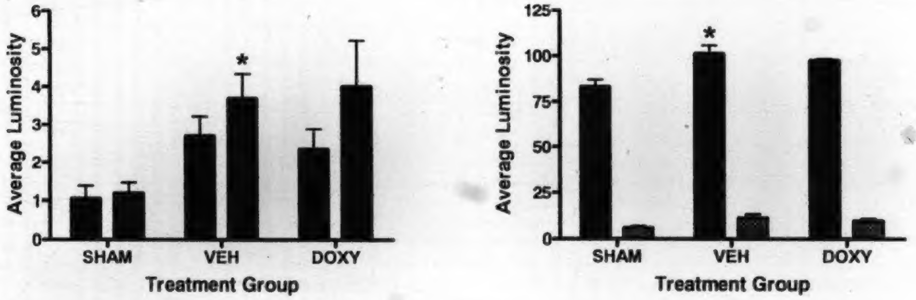
Figure 5.5. Levels of Interleukin-1β (IL-1β), Tumor Necrosis Factor α (TNFα) and Brain Derived Neurotrophic Factor (BDNF), in the Frontal Cortex, Striatum and Hippocampus Ipsilateral to Common Carotid Artery Ligation, 12 hours post Hypoxia-Ischemia (HI). IL-1β and TNFα is significantly increased in the frontal cortex of DOXY-treated pups, 12 hours post-HI. Further, DOXY-treated pups have significantly higher levels of IL-1β compared to VEH-treated pups in the frontal cortex. In the striatum, treatment with DOXY significantly increased TNFα and BDNF compared to both SHAM and VEH-treated pups. Both VEH- and DOXY-treated pups have significantly elevated levels of IL-1β in the hippocampus, 12 hours post-HI. (* = significantly different from SHAM, # = significantly different from DOXY-treated HI pups). Values represent mean ± SD (n=8).

12 hours

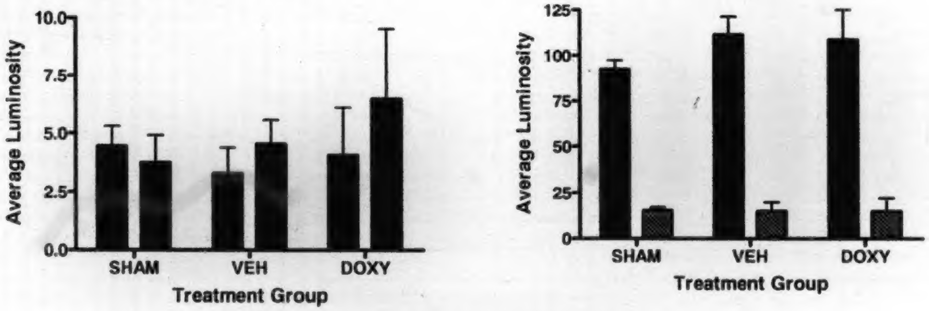
frontal cortex

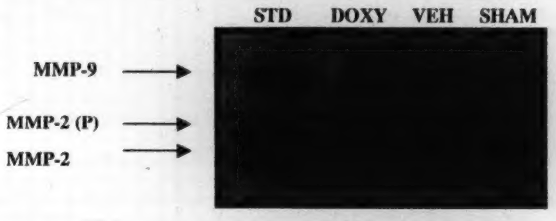


striatum



hippocampus




STD
DOXY
VEH
SHAM
MMP-9
MMP-2 (P)
MMP-2

Frontal Cortex

Figure 5.6. Gelatinase Activity in the Frontal Cortex, Striatum and Hippocampus Ipsilateral to Carotid Ligation, 12 hours Post Hypoxia-Ischemia (HI). Representative histograms and zymograms demonstrating that HI pups treated with either doxycycline (DOXY) and vehicle (VEH) have significantly increased MMP-9 activity and pro-MMP-2 activity in the striatum, 12 hours post-HI. In the striatum, VEH- treated pups exhibited significantly increased activities MMP-9 and pro- MMP-2. (* = significantly different from SHAM. # = significantly different from DOXY-treated HI pups). Values represent mean luminosity ± SD (n=3).

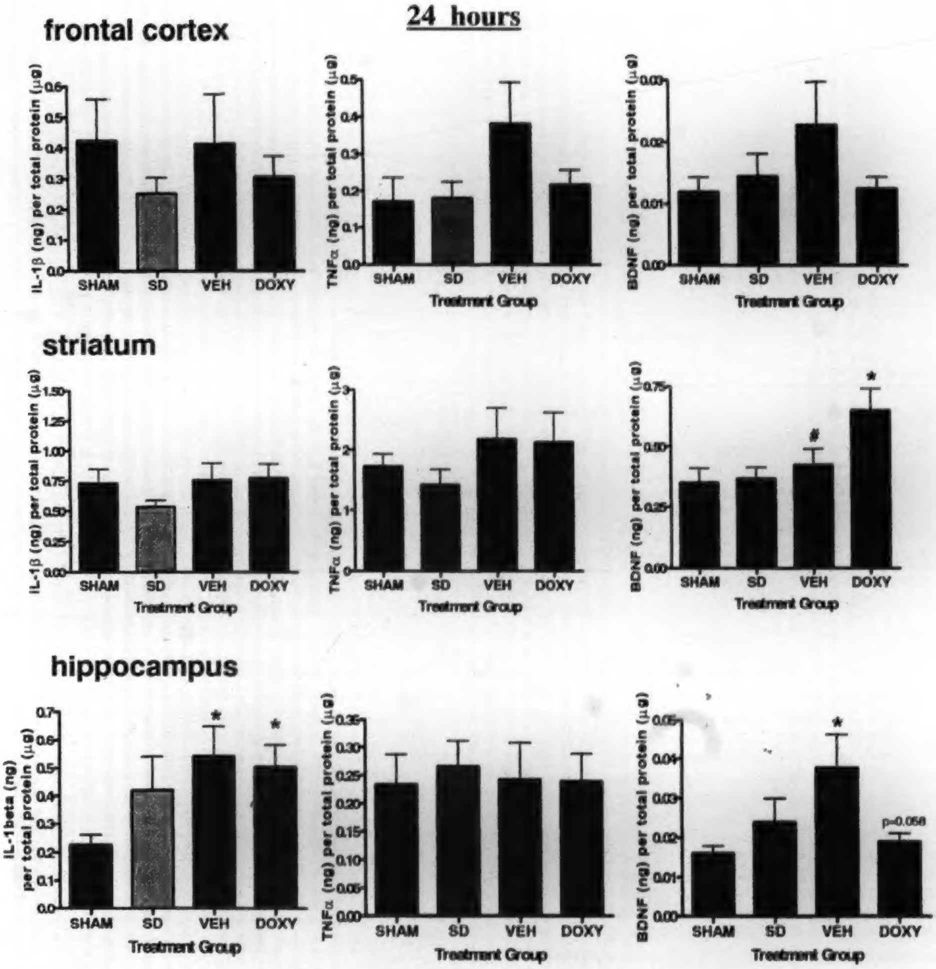
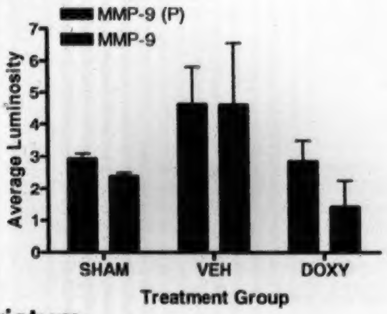
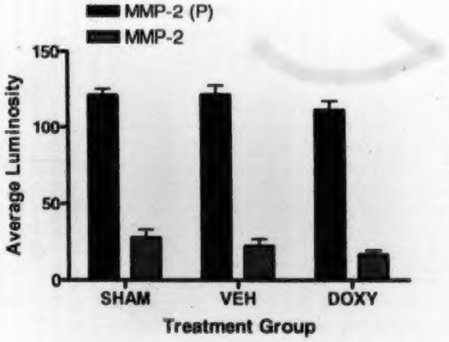


Figure 5.7. Levels of Interleukin-1β (IL-1β), Tumor Necrosis Factor α (TNFα) and Brain Derived Neurotrophic Factor (BDNF), in the Frontal Cortex, Striatum and Hippocampus Ipsilateral to Common Carotid Artery Ligation, 24 hours Post Hypoxia-Ischemia (HI). Twenty-four hours post-HI treatment with DOXY significantly increased BDNF in the striatum, compared to both SHAM and VEH-treated HI pups. In the hippocampus, both DOXY- and VEH-treated pups have significantly elevated IL-1β compared to SHAMs. Also in the hippocampus, VEH-treated pups have significantly elevated BDNF compared to SHAMs. Treatment with DOXY, normalized and decreased BDNF however, this reduction was not significant (p = 0.0581). (* = significantly different from SHAM, # = significantly different from DOXY-treated HI pups). Values represent mean ± SD (n=8).

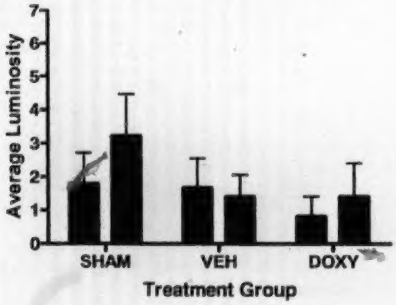
24 hours

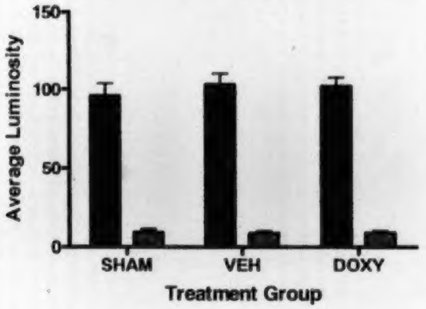
frontal cortex



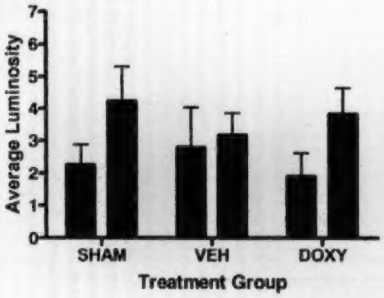


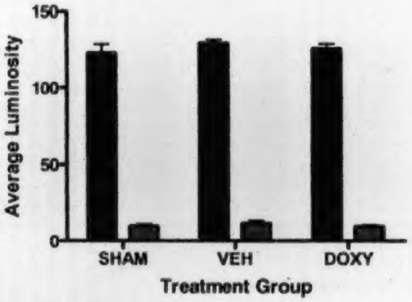
striatum

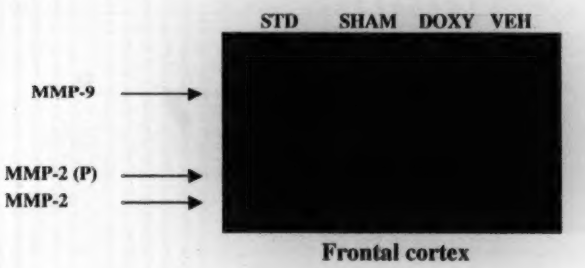




hippocampus






STD
SHAM
DOXY
VEH
MMP-9
MMP-2 (P)
MMP-2

Frontal cortex

Figure 5.8. Gelatinase Activity in the Frontal Cortex, Striatum and Hippocampus Ipsilateral to Carotid Ligation, 24 hours Post Hypoxia-Ischemia (HI). Representative histograms and zymograms indicating that 24h post-HI the activities of MMP-9 and MMP-2 are not significantly different between groups in any region examined. (* = significantly different from SHAM. # = significantly different from DOXY-treated HI pups). Values represent mean luminosity ± SD (n=3).

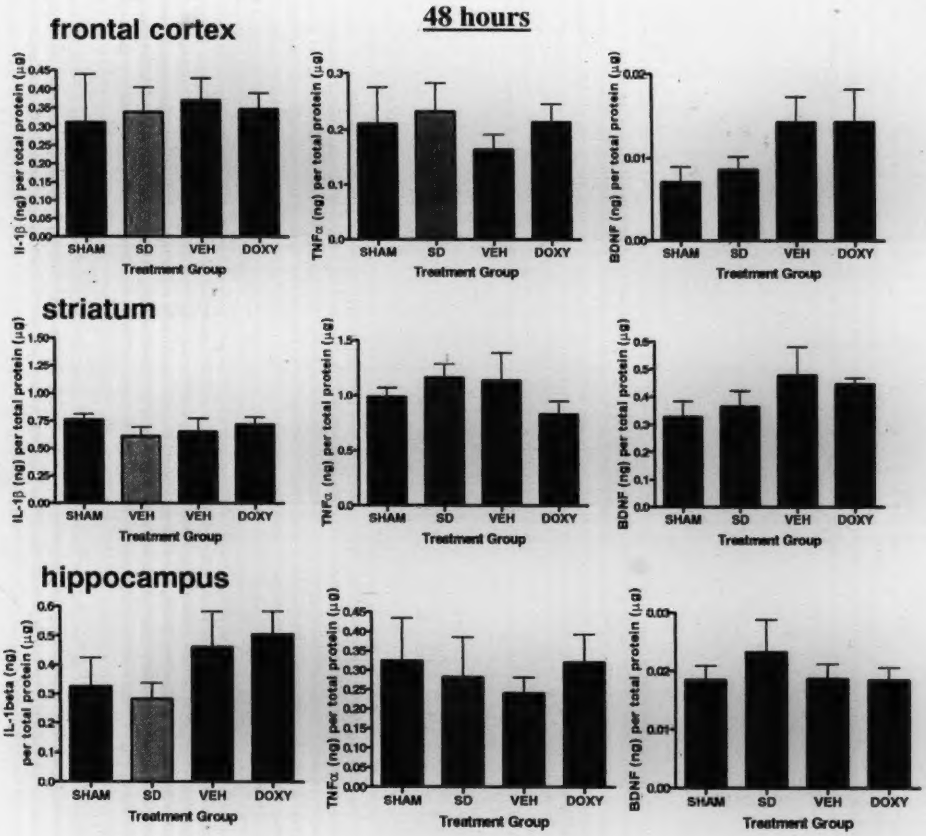
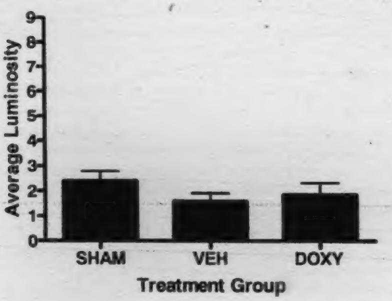
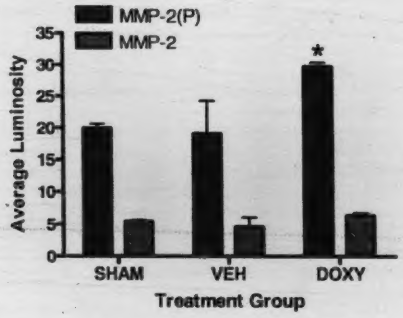


Figure 5.9. Levels of Interleukin-1β (IL-1β), Tumor Necrosis Factor α (TNFα) and Brain Derived Neurotrophic Factor (BDNF), in the Frontal Cortex, Striatum and Hippocampus Ipsilateral to Common Carotid Artery Ligation, 48 hours post Hypoxia-Ischemia (HI). Forty-eight hours post-HI no significant regional changes in IL-1β, TNFα, and BDNF were observed. Values represent mean ± SD (n=8).

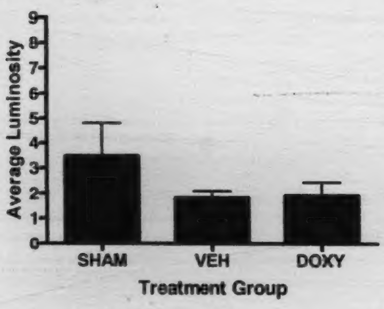
48 hours

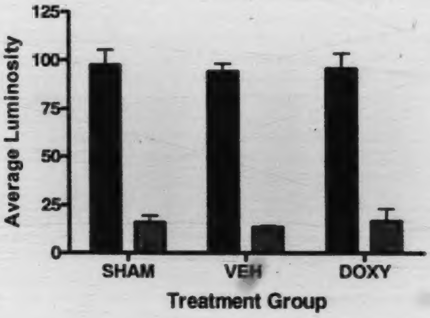
frontal cortex



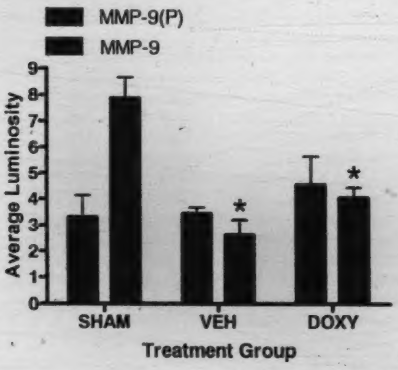


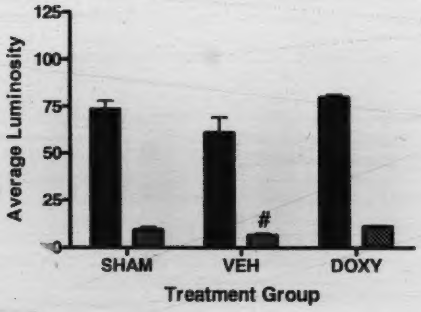
striatum

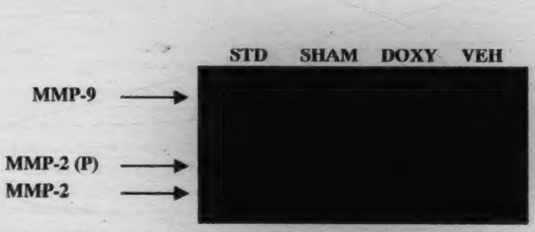




hippocampus






STD
SHAM
DOXY
VEH
MMP-9
MMP-2 (P)
MMP-2

Hippocampus

Figure 5.10. Gelatinase Activity in the Frontal Cortex, Striatum and Hippocampus Ipsilateral to Carotid Ligation, 48 hours Post Hypoxia-Ischemia (HI). Representative histograms and zymograms showing that 48h post-HI, there are significant increases in the activity of pro-MMP-2 in the frontal cortex of vehicle- (VEH) treated pups. MMP-9 has significantly increased activity in the hippocampus of both VEH- and DOXY-treated pups. Also in the hippocampus, DOXY-treated pups have significantly more MMP-2 compared with VEH-treated pups. (* = significantly different from SHAM. # = significantly different from DOXY-treated HI pups). Values represent mean luminosity ± SD (n=3).

7 days

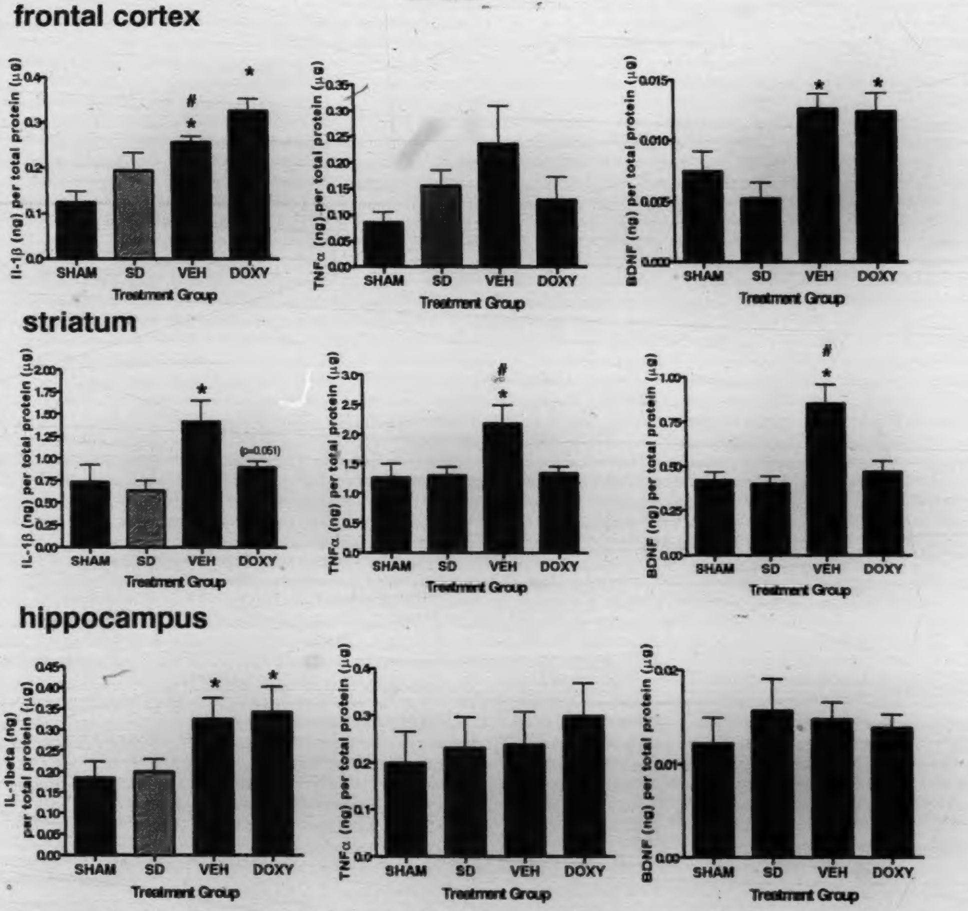
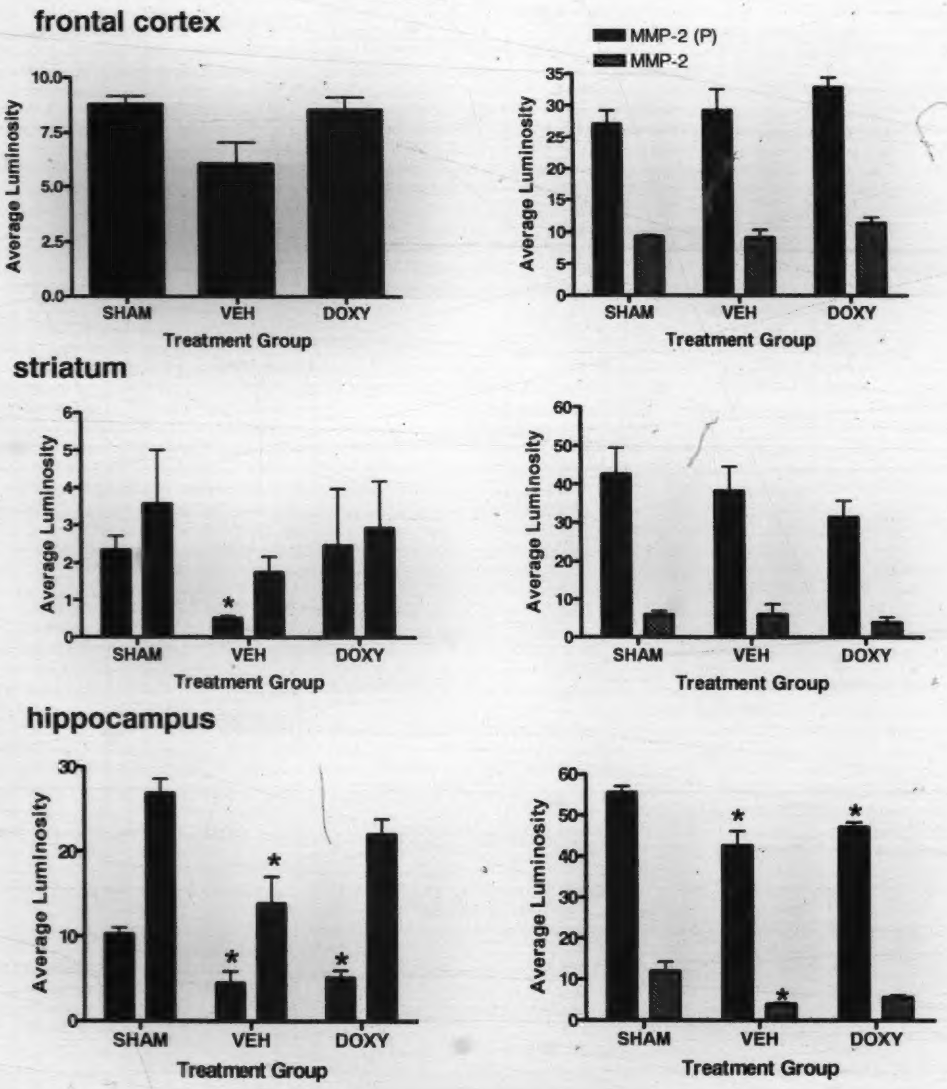
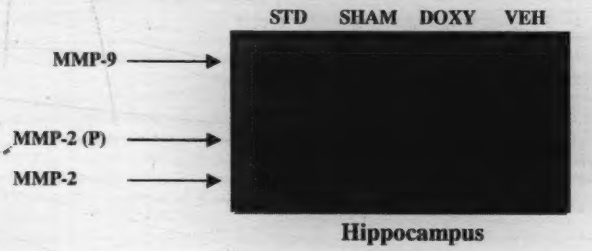


Figure 5.11. Levels of Interleukin-1β (IL-1β), Tumor Necrosis Factor α (TNFα) and Brain Derived Neurotrophic Factor (BDNF), in the Frontal Cortex, Striatum and Hippocampus Ipsilateral to Common Carotid Artery Ligation, 7 days Post Hypoxia-Ischemia (HI). Seven-days post-HI, significant elevations in IL-1β and BDNF in the frontal cortex were observed in both VEH- and DOXY-treated pups. DOXY-treated pups had significantly higher levels of IL-1β compared to those treated with VEH. All HI pups also had significantly increased IL-1β in the hippocampus compared with SHAMs. In the striatum, levels of IL-1β, TNFα, and BDNF were significantly increased compared to SHAM. Treatment with DOXY normalized these increases as it was observed that there was significantly less TNFα and BDNF in the DOXY-treated pups as compared to VEH-treated pups. Treatment with DOXY also reduced IL-1β in the striatum, 7d post-HI although this decrease failed to reach significance ($p = 0.051$). (* = significantly different from SHAM, # = significantly different from DOXY-treated HI pups). Values represent mean ± SD (n=8).

7 days




STD
SHAM
DOXY
VEH
MMP-9
MMP-2 (P)
MMP-2

Hippocampus

Figure 5.12. Gelatinase Activity in the Frontal Cortex, Striatum and Hippocampus Ipsilateral to Carotid Ligation, 7 days Post Hypoxia-Ischemia (HI). Representative histograms and zymograms illustrating that vehicle-treated pups (VEH) have significantly less activity of pro-MMP-9 in the striatum, compared to SHAM. In the hippocampus, VEH-treated pups have significantly increased MMP-9 and MMP-2 activity compared to SHAM. The activities of pro-MMP-9 and pro-MMP-2 are also significantly increased in DOXY-treated pups. (* = significantly different from SHAM. # = significantly different from DOXY-treated HI pups). Values represent mean luminosity ± SD (n=3).

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CHAPTER 6

General Discussion

6.1. Summary of Findings

Overall the studies presented in this thesis establish the complexities of DOXY administration as a putative therapeutic for neonatal hypoxic-ischemic brain damage and emphasize the multifaceted role of microglia in the immature brain after injury. Understanding when inflammation is beneficial and when it is detrimental is crucial to the success and clinical efficacy of anti-inflammatory therapies, and this work highlights the role of DOXY in the treatment of neonatal HI in relation to the dual nature of the inflammatory response. The data presented here are novel, timely and contribute further understanding of the pathophysiology of HI as specifically related to microglia, apoptotic cell death, pro-inflammatory cytokines, amino acid neurotransmitters, and matrix metalloproteinases.

Cumulatively, the findings reported in the preceding chapters indicate that DOXY crosses the blood-brain barrier and is neuroprotective over a 7d timecourse, even following a single dose administered 3h after injury. Not only was this the first report of a neuroprotective and neurorescuing capacity for DOXY in neonatal HI, it also detailed significant neuroprotection even when the pharmacotherapy was administered hours after the initiation of injury. The putative molecular and cellular mechanisms through which DOXY exerts its effects have also been highlighted throughout this body of work and include a reduction in microglial activation, a reduction in the pro-inflammatory cytokines IL-1β and TNFα, and reduced protein expression of cleaved caspase-3. Analyses of the neurochemical data presented in Chapter 2 reveal that HI causes significant, temporal and regional changes in amino acids including glutamate, GABA, alanine, serine, and glycine. Further, those data show that the administration of DOXY significantly reduces pathological increases in alanine, serine and glycine, 4h post-HI. These findings are very novel, as the neurochemical effects of tetracycline administration in a model of neurological injury have not been previously documented. In addition to which, these results expand the effect profile of DOXY to include modulation of amino acid neurotransmitters and as a result, DOXY can no longer be thought of as exclusively an anti-inflammatory or anti-apoptotic compound.

As amino acids, inflammatory cytokines, neurotrophins and apoptotic machinery each have dual nature in brain development and pathology, I sought to investigate the developmental consequences of DOXY administration in HI. The data presented in Chapter 4 show that the administration of DOXY acutely alters cell genesis in the major proliferative zones of the neonatal brain, but that these effects are not sustained beyond 7d post-HI. To elucidate the putative mechanism of action for the cellular changes observed along the investigated timecourse, it was revealed that HI leads to temporal and regional perturbations in IL-1β, TNFα, BDNF and MMP activity. These are the first investigations of regional cytokine and MMP expression after HI with and without DOXY treatment, over an extended acute-subacute timecourse.

6.2. Microglial Activation After Neonatal Hypoxia-Ischemia

The immature brain has been shown in many investigations to be exceedingly susceptible to the consequences of an inflammatory reaction (Anthony *et al.* 1997, Bona *et al.* 1999, Calvert & Zhang 2005). The cellular production and release of chemokines and cytokines, as well as the expression of adhesion molecules in response to HI, potentiates inflammation, damages the endothelial lining of delicate blood vessels and activates a variety of cell death pathways (Saliba & Henrot 2001). Activation of the endothelium is crucial as it facilitates the recruitment and migration of leukocytes into extravascular tissue, and specific molecules included in this process include selectins, β_2 integrins, intracellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), and platelet endothelial cell adhesion molecule (PECAM). In addition to endothelial changes, microglial cell activation precipitates neuroinflammation and neuronal cell death after HI. The first objective of this thesis was to investigate the role of microglial activation in relation to the timecourse of neuroinflammation following HI, and to further our understanding of microglial activation in the context of neuronal survival and death in the developing brain. In corroboration with other studies, results from Chapters 2, 3, and 5 provide evidence suggesting that inflammation plays a major role in the development and propagation of HI brain injury. In contrast to the low numbers of ED-1 positive activated microglia observed in naïve animals, significant increases in activated microglia were documented as early as 1h post-HI. As revealed in Chapter 3, the number and regional distribution of the activated microglia depend on severity of HI injury. In mild HI, which was the paradigm of injury employed throughout the subsequent chapters of this thesis, activated microglia were significantly upregulated in the corpus callosum, hippocampus, cortex and in the reticular nucleus of the thalamus. Irrespective of the dosing regime, DOXY administration significantly decreased the number of activated microglia in these regions. When the time in hypoxia was increased from 60min to 90min, leading to a shift from patchy neuronal cell loss to cystic lesions, the numbers of activated microglia increased exponentially in all areas of the brain such that accurate quantification by classical cell counting was virtually impossible. Nevertheless, administration of DOXY significantly decreased the number of ED-1 positive cells in this HI paradigm.

6.2.1. Regional Susceptibility to Neuroinflammation

As documented in Chapter 5, concomitant with the robust activation of microglia, significant changes in the levels of pro-inflammatory cytokines were observed. Regional levels of TNFα and IL-1β were measured on a timecourse ranging from 3h to 7days post-HI. Significant, regional-dependent increases in these cytokines were observed 3-6h post HI, beginning with an upregulation of IL-1β in the hippocampus at 3h and progressing to an upregulation of TNFα in all three regions examined by 6h post-HI. In each case, these increases were significantly attenuated in the DOXY-treated animals. Blocking IL-1β or administering a recombinant human IL-1 receptor antagonist has been previously shown to attenuate the brain damage caused by HI (Hagberg *et al.* 1996, Martin *et al.* 1994), suggesting that IL-1β plays a major role in the progression of brain damage and cell loss after an HI event. It also highlights one of the many beneficial mechanisms of action that DOXY may have in this type of brain injury. My findings are also supported by previous data showing that increases in IL-1β mRNA levels peak approximately 4h post-HI with a corresponding increase in TNFα shortly thereafter (Szaflarski *et al.* 1995). Although protein amounts as determined by ELISA were determined here, bioactivity of IL-1β has also been shown to increase transiently after HI, reaching a maximum level after 6h of recovery (Hagberg *et al.* 1996). Thus collectively, these data suggest that acute activation of microglia is detrimental after HI and that temporal deregulation of the proinflammatory cytokine response is crucial to the propagation of inflammation associated with neonatal cerebral HI.

In this thesis, stabilization in cytokine levels was observed 48h post-HI. Thus, the timecourse was extended to include a 7d survival endpoint to gain insight on the persistent changes in BDNF, TNFα, and IL-1β and also to observe if there were any sustained differences in neuroinflammation between VEH and DOXY-treated animals. Here, persistent increases in IL-1β were documented in all HI pups, in every region examined. Significant increases in TNFα and BDNF were also observed in the striatum of VEH-treated pups. Although the hippocampus and cortex are classically thought to be the most vulnerable regions to HI injury, persistent increases in both cytokines were only documented in the striatum of VEH-treated pups. As well, it was only in the striatum that the DOXY-treated pups had significantly less TNFα, IL-1β *and* BDNF. Other investigators have highlighted the importance of examining the regional differences in the expression of proteins that interact with death receptor apoptotic cascades (Northington *et al.* 2005). Levels of Fas-ligand and TNFR1 death receptors are increased following perinatal HI in humans, and activation of these receptors is capable of transducing cell survival or cell death signals (Bona *et al.* 1999, Felderhoff-Mueser *et al.* 2000, Graham *et al.* 2004, Northington *et al.* 2001, Northington *et al.* 2005). In the neonatal rat, depending on the region investigated, blocking TNFα has been shown to ameliorate or exacerbate excitotoxic injury (Galasso *et al.* 2000). Specifically, these investigators reported that co-injection of a TNF binding protein with NMDA reduced striatal injury but exacerbated hippocampal damage in the P7 rat. As reported in this thesis, significant changes in TNFα levels were more often observed in the frontal cortex and striatum following the induction of HI. Both of these regions are vulnerable to HI, and neuronal cell loss was observed in each locality. However, in terms of global cell loss after mild HI, the striatum is least affected compared to the dorsal lateral cortex, dentate gyrus, and thalamus. In most cases, increased striatal levels of TNFα were attenuated by DOXY, which likely reflects a beneficial reduction in neuroinflammation and by extension, less activation of TNF receptors, potentially leading to dowregulation of extrinsic apoptotic cascades and increased neuronal survival.

There are also developmentally-regulated regional brain differences in the susceptibility to HI injury and territories at highest risk for injury are those that have less circulation, high metabolic demands, and a high density of excitatory neurons (Calvert & Zhang 2005, Qiu *et al.* 2007). For example, the cornu ammonis (CA) regions of the hippocampus are relatively resistant to HI in the immature brain and do not develop their characteristic vulnerability to ischemia until P13-P21 in rats (Qiu *et al.* 2007, Towfighi *et al.* 1997, Zhu *et al.* 2005). This is in stark contrast to the DG which appears to be more vulnerable in the immature brain than in the juvenile or adult (Qiu *et al.* 2007, Towfighi *et al.* 1997). In this thesis, histopathological analyses performed after a 7d survival period corroborates these findings, especially in animals that were subject to moderate HI. In certain cases, complete ablation or absence of the DG was observed in VEH-treated pups with the relative sparing of CA1. Interestingly, consistent cell loss in CA2 after HI was also noted. The region-specific deregulation in cytokines, especially the specific increases in TNFα, IL-1β and BDNF observed from 6h-7d post-HI, may be related to regional susceptibility dictated by the response to and propagation of inflammation. When the MMP data in Chapter 5 are considered, these changes could also represent developmental regional differences related to structural rearrangement, myelin formation, angiogenesis and/or plasticity. However, given that DOXY administration restored levels of TNFα, IL-1β, BDNF and MMP activity to those seen in SHAM animals, it is likely that its anti-inflammatory effects were primarily responsible for the improved pathology.

6.2.2. Persistence of Neuroinflammation

The timeline of the inflammatory response after HI has been investigated in other laboratories. In addition to increased expression of TNFα and IL-1β, increased expression of β_2 integrins, along with an accumulation of neutrophils often follow upregulations in macrophage inflammatory protein (MIP)-2 after HI (Calvert & Zhang 2005). This is significant because increased expression of these molecules and the β-chemokines such as MIB-1β and MIP-1α are followed by additional accumulation of microglia/macrophages, $CD4^+$ and $CD8^+$ lymphocytes, and natural killer (NK) cells in infarcted areas (Anthony *et al.* 1997). This indicates complete activation of the neuroinflammatory response as well as the recruitment and activation of peripheral immune cells. Twelve hours after HI, many isolated or aggregated neutrophils can be identified within blood vessels, attached to vessel walls, and at various stages of migration through vessels into infarcted regions (Bona *et al.* 1999). On the other hand, NK cells start to accumulate at time points when neutrophils are not seen in HI tissue. In contrast to the vast numbers of CD8$^+$ cells found in and around ischemic infarcts 3-7d post-ischemia in adult animals, neonatal cerebral lymphocyte activation involves mainly CD4$^+$ cells, and discrete inductions of CD8$^+$ cytotoxic/cytolytic T cells are only observed 24-72h post-HI (Bona *et al.* 1999, Schroeter *et al.* 1994). These data strongly suggest that microglial activation occurs earlier than lymphocyte infiltration in HI because CD4$^+$ T cell activation can only be induced by an antigen presenting cell (microglia/macrophage) displaying the MHC class II together with the antigen (Bona *et al.* 1999).

Further, it has also been reported that activation of microglia/macrophages, CD4$^+$ lymphocytes and astroglia can persist for 35 days, signifying that chronic inflammation is observed after an HI insult (Anthony *et al.* 1997). Data collected from my pilot studies indicate that significant numbers of activated microglia do not exist 35d post-mild HI (Figure 6.1). However, a significant degree of astrocytosis, as indicated by hypertrophic cell bodies and increased expression of GFAP, is observed (Figure 6.2). In addition, more reactive astrocytes are found in the hippocampus and callosal white matter of VEH- and DOXY-treated pups compared to the degree of astrocytosis observed in the frontal cortex (data not shown). In the experiments of long-term survival, I administered repeated doses of DOXY (10 mg/kg/day) for 7d following the HI injury. Thirty-five days after the insult, DOXY-treated pups have significantly more reactive astrocytes in the hippocampus compared to SHAM animals (data not shown). These findings presented an interesting question regarding the significance and purpose of this reactive astrocytosis. Are these data simply corroborating the findings by other investigators and attesting to the persistence of inflammation, even in the absence of activated microglia, or could this be an indicator of repair or recovery from HI? As depicted in Figure 6.3, I found that a significant proportion of the astrocytes in the hippocampus were expressing IL-1β as evidenced by GFAP/IL-1β co-localization, whereas neurons were not (Figure 6.3). This is yet another indication of the temporal and regional changes in IL-1β after HI. However, whether this finding signifies the continual propagation of the inflammatory response or is a potential reparative effect remains to be seen, and IL-1β would most certainly have to be quantified in further experiments and the levels in DOXY- and VEH-treated pups compared. Even though the immature brain has been shown to respond to IL-1β with a stronger and more rapid recruitment of leukocytes compared to the adult brain, more experimentation with regard to immune cell signaling and sustained interactions between peripheral and central inflammation after HI also needs to be undertaken (Bona *et al.* 1999, Anthony *et al.* 1997).

Caution should be exercised when making causative links based on the temporal relationships presented here, and future studies are necessary to understand the cellular origin of BDNF, TNFα and IL-1β leading up to 35d post-HI and the putative developmental impact of chronic astrocytic IL-1β expression. However, as cytokines are produced in minute amounts and exert biologically relevant effects at extremely low concentrations (Bona *et al.* 1999), the data presented in Chapter 5, especially in the context of the shift in regional profiles after HI, is highly relevant to understanding the relationship between cell death and cell survival post-HI.

6.3. DOXY is Neuroprotective in an Animal Model of Neonatal Hypoxia-Ischemia

Overall, these data represent an acute and sub-acute snapshot of inflammation after neonatal HI, and the consequences of DOXY administration. As DOXY was shown to have anti-inflammatory properties, the second objective of this thesis was to study the putative neuroprotective properties and developmental consequences of DOXY administration. As described in Chapter 2, neuroprotection in DOXY-treated pups was observed as early as 4h post-HI. In pups treated with DOXY, there were significantly more NeuN-positive neurons, and fewer Fluoro-Jade positive cells observed. Pups treated with DOXY exhibited decreased regional expression of cleaved caspase-3, in addition to the decrease in ED-1 positive microglia. Accompanying this decrease was a concurrent upregulation of pro-caspase-3 protein levels, possibly indicating that the administration of DOXY was preventing cleavage from the pro- to the active-form of caspase-3. When survival from HI was extended, the ability of DOXY to provide both neuroprotection and neuronal rescue was noted. This investigation was unique, as not only were pups pre-treated with DOXY (a neuroprotection regime), but separate groups of pups were run such that DOXY was administered immediately after HI or 1, 2, or 3h post-injury (a neurorescue paradigm). The data collected proved that DOXY was capable of protecting or rescuing neurons from HI-injury, as even when administered as a single dose 3h post-HI. Decreased cleaved caspase-3 protein expression and significantly fewer activated microglia accompanied the observed increase in neuronal survival. When these data from Chapter 3 are combined with data from Chapter 5, it is clear that DOXY's neuroprotective properties exist independently of *global* pro-inflammatory cytokine decreases and MMP activity. Further, I provide evidence to indicate that the threshold for DOXY's anti-inflammatory and anti-apoptotic effects is lower than the threshold required for MMP inhibition.

6.3.1. Apoptotic Cell Death

Throughout, this thesis has demonstrated a consistent upregulation of cleaved caspase-3 after HI and an amelioration of this phenomenon with the administration of DOXY. As discussed previously, the activation of caspase-3 can occur via cytokine-mediated receptor activation or through an alteration of the mitrochondrial membrane potential, leading to release of cytochrome c. It is assumed that both intrinsic and extrinsic apoptotic cascades are active in these studies, as upregulation of TNFα after HI was observed, and other investigators have confirmed upregulation of other death receptor ligands (i.e. Fas) in concert with mitochondrial disruption related to energy failure and oxidative stress. It has been proposed that the contribution of apoptosis to neuronal degeneration is most significant in the delayed phases of injury and in milder forms of neonatal HI (Northington *et al.* 2005). I found significantly more cleaved caspase-3 positive cells in the brains of animals subjected to moderate-HI, with overall lesion size being much larger in these animals compared to those subjected to mild injury, even at the earliest time points examined. To achieve the magnitude and volume of cell loss documented in the pups subject to moderate HI, necrotic cell death would most certainly have a major role. Further, significantly more Fluoro-jade positive cells were observed in moderate HI, and Fluoro-jade staining is believed to operate independent of caspase machinery. Apoptosis occurs on a much slower and protracted timecourse than necrosis, and alone would have been unable to account for all of the cell loss occurring in such a short time. My data corroborate the notion that apoptotic cell death or, at the very least, caspase-dependent cell death, plays an extended role in milder lesions, as the caspase activation was present throughout the week long timecourse, was observed in many cell types, and was even found in the absence of global volume loss and substantial infarcts.

Certain structural characteristics of dying neurons observed in the first 24h after neonatal HI corroborate the notion that apoptotic mechanisms are acutely operative, even though necrosis is likely the driving force behind the initial amount of cell loss after HI. However, a phenotypic diversity of cell death has also been detailed to suggest that a mixture of apoptosis, necrosis and intermediate forms of cell death best described as a "continuum" phenotype exists following HI in the neonate (Northington *et al.* 2005, Blomgren *et al.* 2001). This complexity suggests that apoptosis inhibitors alone will be inadequate to ameliorate the early brain damage following neonatal HI and may simply push cells toward necrotic cell death as seen *in vitro* when caspase inhibitors are administered following hypoxia (Northington *et al.*

2005, Formigli *et al.* 2000, Portera-Cailliau *et al.* 1997). Just as the nature and extent of neuronal injury are important factors that dictate how microglia behave in response to ischemia, the fundamental mechanism driving the necrotic-apoptotic cell death continuum is thought to be gradations in the responding cells to stress, the relative speed of each pathway and the opposing action of survival mechanisms (Unal-Cevik *et al.* 2004). In adult animals, a mixed phenotype of cell death is more common in transient ischemia compared with permanent ischemia, possibly because more cells have a chance to activate apoptotic machinery before the collapse of energy metabolism and loss of membrane integrity (Unal-Cevik *et al.* 2004). This is highly relevant to neonatal HI, as pure ischemic strokes occur much less frequently than transient HI events related to pregnancy and this could explain why a mixed phenotype of cell death is more common in the immature brain.

Regardless of the phenotype of cell death that exists after brain injury, apoptosis is critical for normal brain development and function, and is required for synaptic consolidation, myelination and pruning. The most dramatic evidence for role that caspases play in development is provided by investigations using caspase-3 and caspase-9 knockout mice. These mice exhibit severe cerebral malformations, including marked ventricular zone expansion, exencephaly, and ectopic brain growth (Kuida *et al.* 1996, Kuida *et al.* 1998, Northington *et al.* 2005). These experiments speak to the effects of permanent blockade of caspase activity and are not truly relevant to the question of whether transient pharmacologic inhibition of caspases in neonatal HI would be deleterious (Northington *et al.* 2005). However, they do provide important insight into the developmental consequences of interfering with apoptosis in the immature CNS. Recent evidence suggests that chronic pharmacologic inhibition of the caspases simply pushes apoptotic machinery to upregulate caspase-independent cell death pathways (West *et al.* 2006). Specifically, long-term inhibition of cleaved caspase-3 during development leads to upregulation of cleaved caspase-3 independent cell death pathways and increases vulnerability of the neonatal brain to HI (West *et al.* 2006). Collectively, these data raise some important questions about the use of DOXY in this injury. As previously discussed, a single dose of DOXY is capable of significantly decreasing cleaved caspase-3, in a sub-acute survival setting. However, it is clear from previous studies using tetracyclines in neurological disease and the data provided in chapters 2, 4 and 5, that DOXY is not purely an anti-apoptotic drug. Thus, concerns with regard to complete downregulation or absence of cleaved caspase-3 is likely a minor issue, although it is duly recognized that many questions still remain surrounding these therapeutic strategies in relation to the cross-talk between different cell death pathways, especially caspase-independent cell death. Most importantly though, acute inhibition of apoptosis is a completely distinct phenomenon from chronic inhibition or permanent absence, and it is clear from the data provided in this thesis that the administration of DOXY certainly does not lead to *absolute* inhibition of cleaved caspase-3. Although hypothermia, caspase inhibitors, anti-excitotoxic drugs and anti-inflammatories have proven significantly neuroprotective in many animal models of neurodegeneration, including in the experiments described here, it is unclear whether these treatment strategies simply preserve cells functioning in a sub-optimal state and/or *delay* the inevitable neurodegeneration. Further, it is unclear whether the neuroprotective/neurorescuing effects persist chronically and lead to long-term improvement after HI. Additionally, the implications of keeping quasi-injured neurons alive with therapies such as DOXY are unknown and thus further experiments involving long-term survival are needed.

Preliminary studies that I have performed suggest that HI leads to a sustained upregulation of cleaved caspase-3, when examined 35 days after insult (Figure 6.4), and corroborates earlier data suggesting apoptosis plays an extended role in mild HI. As mentioned previously, ongoing caspase-dependent cell death and injury evolution after HI have been demonstrated in numerous investigations. Using the same animal model of HI as described in this thesis, active caspase-3 has been shown to be reduced from very high levels in P7, to moderate levels in P15 and P26, to barely detectable levels at P60 (Hu *et al.* 2000). In relation to the long-term consequences of mild versus moderate and severe HI, it is hypothesized that although cerebral atrophy is not evident 2 weeks after mild HI, cell death evolves significantly over the following 6 weeks such that by 8 weeks post-HI there are no neuropathologic differences between mildly and severely injured animals (Geddes *et al.* 2001). Again, this highlights the long-term contribution of apoptotic cell death and persistent neurodegeneration in neonatal HI. The pilot data I have collected in relation to 35 day (5 week) survival from mild HI confirm that there is neuronal loss and significant cellular disorganization in the cortex, hippocampus and thalamus (Figure 6.5). Although these data do not indicate severe cerebral atrophy, persistent cystic lesions or large areas of infarct are present in this survival setting. However, cortical disorganization is especially evident in VEH-treated pups compared to DOXY-treated animals, as layering is abnormal, neurons are misshapen, neuropil is loose and areas of patchy cell loss are unmistakable. These data also indicate that there are significantly more neurons in medial CA1 of the hippocampus contralateral to carotid artery ligation in both DOXY- and VEH-treated pups compared to SHAM (Figure 6.5). Further investigation into the significance and full extent of these findings is clearly required, as they may be indicative of dysfunctional neuronal migration, contralateral compensation and cellular reorganization and/or persistently increased neurogenesis.

6.3.2. Cell Genesis and the Birth of New Neurons

As it was established in Chapter 2 and Chapter 3 that DOXY was neuroprotective, I sought to investigate the acute impact of DOXY administration on cell genesis, specifically neurogenesis. An acute survival paradigm was chosen because injury-induced proliferation, including neurogenesis, is most pronounced during the earliest phases after HI (Qiu *et al.* 2007). The question as to whether DOXY affected neurogenesis was crucial for two reasons: 1) Inflammation and apoptotic cell death have dual roles in development and pathology, and inhibition of neuroinflammation could have a negative impact on the genesis and proliferation of new cells, and 2) The clinical value of any putative therapy for a childhood/neurodevelopmental disorder rests on the demonstration that the trajectory of development post-injury is not altered by the treatment (i.e. one developmental disorder will not be exchanged for another). Before the studies outlined in Chapter 4, the effects of the tetracycline antibiotics on cell genesis, DNA synthesis and impact on eventual neurogenesis had not yet been studied. Although more work is still needed, my data suggest that the sub-acute timecourse of cell genesis after HI is not affected by the administration of DOXY. Overall, these studies establish that although DOXY administration reduces the number of BrdU-positive cells in the SVZ 6h post-HI, there are no sustained reductions in BrdU immunoreactivity at 48h or 7d post-HI in either the DG or SVZ. To extend this finding, the phenotype of the new cell progeny after HI was investigated. Although neurogenesis takes place over a protracted trajectory, my results indicate that 7d after HI the majority of new cells are negative for glial markers but positive for nestin. At this time point, BrdU/NeuN positive cells were also observed, indicating that although the majority of the new cells may be undifferentiated at this time, some are indeed of mature neuronal lineage. Work done by other investigators corroborate these data, as many have found that proliferating cells in the medial SVZ after perinatal HI express markers of immature multipotent precursors (nestin), but not markers of mature neurons (NeuN) (Felling *et al.* 2006).

One week following HI, some proliferating cells appear to migrate from the SVZ into damaged striatum and differentiate into phenotypically mature striatal neurons (Arvidsson *et al.* 2002, Felling *et al.* 2006, Parent *et al.* 2002). These phenomena imply that deficient neuronal function might be repaired by replacing lost neurons and this is basis for the development of restorative therapies for brain injury (Qiu *et al.* 2007). In this aspect, the dual nature of inflammation is again stressed. Even though the growth factors, cytokines, and MMPs released by activated microglia have been shown to have important roles in the regulation of cell differentiation, cell number, synapse formation, axonal guidance and neuroblast migration (Aarum *et al.* 2003), it is recognized that inflammatory insults likely compromise the survival of newly formed neurons as well (Ekdahl *et al.* 2003). In adult animals, microglial activation has been shown to impair both basal and insult-induced hippocampal neurogenesis (Ekdahl *et al.* 2003). Specifically, activated microglia can be found in close proximity to newly formed cells, and neurogenesis can be inhibited depending on the degree of microglial activation (Ekdahl *et al.* 2003). In these studies, the administration of minocycline restored hippocampal neurogenesis impaired by inflammation without affecting the degree of neurogenesis observed in control animals. These investigators also found that the actions of minocycline were not mediated via stimulation of cell proliferation because no change in Ki-67 immunoreactivity (a marker of proliferating cells in G1, S, G2 and mitosis) was observed. Minocycline also did not alter the degree of neuronal differentiation from the proliferating cells because the percentage of BrdU-positive cells double-labeled with NeuN was similar in the MINO- and VEH-treated rats (Ekdahl *et al.* 2003). Although the aforementioned studies were carried out in adult animals and not in relation to ischemia, my data essentially corroborate these findings. The administration of DOXY did not result in a sustained change in cell proliferation after HI, and there were no differences in acute neuronal differentiation between treatment groups in the timecourse investigated. The observation that the administration of DOXY does not change cell genesis, is certainly not a negative finding. Any observation that indicated that DOXY was persistently increasing or decreasing cell genesis would have been interpreted as a severe side effect of this compound. The situation in neonates is extremely complicated because endogenous neurogenesis is naturally occurring and then HI is superimposed; thus it would be disadvantageous to administer a therapy that interferes with the cell cycle and/or endogenous repair. Further, the destruction of neuronal stem cells by any means during the perinatal period will hinder the continual production of new cells and the loss of stem cells during this critical period will undeniably have long-lasting adverse effects (Levison *et al.* 2001). Obviously, extension of the timecourse is required to fully understand the effects of DOXY on neuronal differentiation, chronic neurogenesis, neuronal migration and long-term survival of newly born cells.

Despite the trend in BrdU immunoreactivity over an entire week in both the DG and SVZ, DOXY did significantly reduce the number of BrdU-positive cells in the SVZ 6h post-HI. Although this reduction was not sustained, it is almost certainly an effect related to DOXY itself, as even SHAM animals treated with DOXY had significantly fewer BrdU positive cells compared to SHAM animals treated with saline. This finding is likely related to high levels of DOXY in the brain and thus, maximal impact on microglial activation. Support for this hypothesis is provided in Chapter 5, in which DOXY-treated pups had significantly less IL-1β and TNFα compared to VEH-treated pups in the striatum at 6h post HI. Also in support, other investigators have suggested that activated microglia have a deleterious effect on newly formed neurons mediated by the action of IL-1β, TNFα, NO and/or reactive oxygen species (Ekdahl *et al.* 2003). With respect to my data, of special interest is the reduction in

TNFα observed with DOXY administration, as this decrease occurred in the absence of TNFα changes in any other experimental group, most notably the VEH-treated HI pups. To rephrase, there were no significant differences in TNFα levels between SHAM, SHAM pups treated with DOXY (SD) and VEH-treated HI pups at this time point. This is in stark contrast to the trend observed with IL-1β, where VEH-treated pups had significantly increased levels of IL-1β compared to SHAM and SD pups, which was attenuated following DOXY treatment. There were also no DOXY-dependent changes in MMP-2 or MMP-9 activity at this time point. These results and the focus on TNFα 6h post-HI in the striatum must be interpreted with caution because it is clear that there were no significant differences in the cytokines when SHAM and SD pups are compared. Thus, other factors in addition to TNFα and IL-1β are likely responsible for the changes observed, as the data presented in Chapter 5 cannot fully account for the DOXY-dependent reductions in BrdU immunoreactivity. When these data are taken together, the temporal importance of microglial activation and the dual nature of cytokines are effectively demonstrated. Perhaps extension of this investigation to include other neurotrophins and growth factors would be beneficial to understand the reason for the DOXY-dependent reduction in the number of BrdU positive cells. Microglia have a marked neuroprotective potential, serving as an endogenous pool of molecules such as IGF-1, fibroblast growth factor-2 (FGF-2) and EPO (Lalancette-Hebert *et al.* 2007). Each of these molecules have important roles in cell proliferation in the SVZ (Iwai *et al.* 2006, Kokaia & Lindvall 2003) and serve as potentially important sources of trophic support for ischemic and proliferating cells (Lalancette-Hebert *et al.* 2007). Thus, the extension of this study to include other molecules involved in cell survival would be beneficial for a more complete understanding of the effects of microglial inactivation on cell genesis.

Regional analyses of the cytokines, in the context of cell genesis, were vital because previous studies have shown that cell fate of multipotent progenitors in the SVZ is partly determined by specific environmental cues (Levison *et al.* 1993). For example, SVZ cells can migrate through adjacent white matter in gray matter to colonize the cortex, striatum and callosal and periventricular white matter (Levison *et al.* 1993). Glial progenitors that remain in the white matter have largely been shown to become oligodendrocytes, while those that settle in gray matter are capable of developing into different types of glia including oligodendrocytes, microglia and astrocytes. This diverse differentiation indicates that the fate of a glial progenitor cell is mediated by multiple factors. It also indicates the important roles that diffusible factors such as TNFα, IL-1β, BDNF and other signaling molecules, released by activated microglia and other mature cells in a specific brain region, have in the cellular communication, support and survival of new and migrating cells.

6.4. Clinical Translation

Despite major advances in the understanding of the underlying mechanisms in HI insults, an effective treatment plan or strategy to reduce associated brain injury is still not available (Calvert & Zhang 2005). Moreover, successful benchwork treatments have not translated to the clinic because of: 1) The complex pathology of HI brain injury in neonates; 2) The difficulty in defining the onset, duration and severity of injury; 3) The side effects of experimental drugs; and 4) The inability of experimental treatments to target multiple pathophysiological cascades (Calvert & Zhang 2005). It is possible that DOXY may have a clinical role, as it has multiple mechanisms of action and a lesser side-effect profile when compared to other drugs in its class and compared to other HI therapies. However, pharmacologically speaking, DOXY is a "dirty" drug. It has multiple mechanisms of action and at the very least, cumulatively affects inflammation and apoptosis on many levels and is capable of modulating amino acid neurotransmitters. These properties could be desirable, as previous therapies investigated for the treatment of HI brain damage have been heavily criticized for interfering with only one aspect of the pathophysiological cascade. This is especially relevant given the molecular and physiologic cross-talk between pathophysiologic HI pathways.

Because the timing of cell death and other biochemical events following HI insult have been extensively mapped out, prospective therapies should be tailored to such events. (Calvert & Zhang 2005). Even though the use of single therapies in an experimental setting has proven invaluable to mapping the pathological cascade after insult, single treatments have not been clinically effective (Calvert & Zhang 2005). Thus, there also needs to be a movement toward the use of multiple "cocktail" therapies that target different aspects of the pathophysiology associated with HI. For example, initial therapies could target the earliest pathological events (i.e. excitotoxicity, reperfusion and free radical injury) and attempt to extend the therapeutic window (i.e. hypothermia). Later therapies could then be used to combat secondary energy failure and events that cause progressive cell death and lesion expansion. Figure 6.6 represents my conception of a putative treatment paradigm for the brain damage associated with HI, involving experimental therapies currently being investigated for use in the clinic and their pathophysiologic targets. With respect to the role that DOXY or similar pharmaceuticals may play in a cocktail approach, my data indicate that this therapy could be initiated as soon as injury is recognized and continued through the peak of the inflammatory response, even though further investigation is required to delineate the shift from pathology-related inflammation to recovery and repair. This is vital for maintaining the innate trajectory of brain development post-HI.

In summary, the role that inflammation plays in propagation of brain damage following HI is significant, and many of the cellular elements crucial to this process have been highlighted in this thesis. DOXY has been used as a tool to manipulate microglial activation after HI and, as a result, considerable data have been gathered to suggest that DOXY might be a useful therapeutic for combating HI-associated changes in neuroinflammation, amino acids and neurogenesis. Neonatal HI presents a unique set of challenges related to the age of the patients and developmental arcs. Thus, as stated so eloquently by Northington *et al.* (2005), "investigators in this field bear a unique burden as they delve into the application of therapies to the developing brain, to not interfere with normal brain development and normal compensatory mechanisms in a potentially harmful manner" (Northington *et al.* 2005). It is with that thought in mind that investigators must pursue safe and effective treatment strategies in the attempt to reduce the medical, emotional, physical, social, societal and financial burdens associated with neonatal hypoxic-ischemic brain injury.

6.5. Future Directions

Although work done in this thesis has both expanded the current understanding of the anti-inflammatory properties of DOXY and suggested a putative role for its use as a therapeutic in neonatal HI, more information is needed to address the molecular mechanisms and pathophysiological properties which underlie HI-related neuronal dysfunction. The contribution and role of peripheral inflammatory cells such as neutrophils and T-cells after HI and in response to treatment with DOXY, is warranted as the infiltration of systemic immune cells after injury were not addressed in the work presented here. As such, most of the future directions of these thesis projects involve an expansion of the timecourse presently investigated, with additional cell-specific markers. Long-term outcome studies would allow a therapy such as DOXY to be tested for neuropathologic, biochemical and functional outcomes, and further safety in the immature brain (Northington *et al.* 2005). In addition to studies into the long-term consequences of DOXY administration on neuronal survival and sustained neuroprotection, further research into the full neurodevelopmental effects of this strategy need to be undertaken. Extension of this aspect of my work would require survival up to 8 weeks post-HI and would include extensive investigation into persistent neurogenesis, the sustained survival of new cells, the arborization of dendrites, synapse number and examination of cortical layering and organization. By association, studies into neuronal migration and differentiation would also be required. Although other animal models, including those in fetal sheep and piglets, are well suited for study of acute and subacute metabolic and physiologic endpoints, I would continue to use the neonatal rat model despite the fact that the P7 rat pup is quite immature and the small size of the animal generally prohibits intensive monitoring of relevant physiological variables during or after the HI insult (Northington *et al.* 2005, Ginsberg & Busto 1989). Other investigators have criticized the model described here for having a non-clinical distribution of injury, with patterns of injury recapitulating a mixture of what is observed clinically in global asphyxia and true ischemic stoke (Ginsberg & Busto 1989). However, most infants who sustain a HI brain injury do exhibit a mixed phenotype of brain damage and most fetal injuries do not involve the formation of thrombi or emboli. More commonly, injuries are related to coiling or wrapping of the umbilical cord and dysfunction in the maternal-placental-fetal system, leading to transient, and/or incomplete HI. Further, studies involving rodents are more conducive to long-term neurological and behavioural outcome experiments, which are equally, if not more important than the cellular neurodevelopmental investigations described above. The demonstration that a neuroprotective compound leads to functional improvement is crucial to transition to human application. Examples of neurobehavioural tests that could be performed include the Morris water maze, rota-rod, and Montoya staircase. Therefore, in addition to further pathologic evaluation over a protracted timecourse, behavioural outcomes would also be evaluated following DOXY treatment.

Recent evidence has suggested that the tetracycline antibiotics have a much shorter half-life in rodents compared to humans (Yong *et al.* 2004). Thus, it is quite probable that the animals in the studies presented here were under-dosed. Although it is well accepted that the anti-inflammatory properties of the tetracyclines occur at doses much lower than those required for their antimicrobial effects, and I document neuroprotection associated with a once daily administration of DOXY at 10mg/kg, it would be beneficial to study different dosing regimes in neonatal HI. Perhaps of most relevance would be different repeated dosing regimes under long-term survival conditions. Not only would this elucidate further consequences of microglial inactivation, it would also provide additional information for clinical translation. My preliminary evidence suggests the persistence of inflammation and cell survival 5 weeks after HI when repeated doses of DOXY were given once daily for 7 days. With respect to half-life and clinical translation, it would be interesting to investigate the administration of DOXY b.i.d. (twice daily) or t.i.d (thrice daily) over the first 3 days post-HI. Presumably, this would lead to a stronger anti-inflammatory effect during the time over which the HI-associated inflammatory response is strongest and likely most detrimental. As mentioned previously, extending the use of DOXY many days or weeks post-injury would likely inhibit the beneficial actions of microglia in injury repair and recovery.

As described in Chapter 2, I investigated changes in cerebral amino acids over a 4 hour time course. To extend the findings in that chapter, the next step would be to measure cerebral lactate and pH. This would generate data to address my hypothesis that the reduction in alanine observed following DOXY administration is beneficial and related to the biochemical pathways connecting alanine and lactate, and by extension pH. Mechanistically and cellularly, the findings in this thesis could be expanded by investigating changes the p38 MAPK cell signaling pathway. This pathway is activated by death receptor activation, cytokines, and growth factors (i.e. TGFβ), and p38 is the major MAPK involved in the induction of NO and TNFα. The exact mechanism of action of DOXY has not yet been documented (i.e. what exactly does DOXY do to microglia that leads to their inhibition), but p38 MAPK has been implicated in the beneficial effects of minocycline and DOXY has been shown to reduce p38 MAPK in lung epithelial cells (Cui *et al.* 2008, Hoyt *et al.* 2006, Hua *et al.* 2005, Tikka *et al.* 2001a, Tikka *et al.* 2001b, Tikka & Koistinaho 2001). Further investigation using DOXY in comparaison to the p38 MAPK inhibitor SB203580 would provide valuable information regarding the mechanism(s) by which DOXY inhibits microglial activation in a model of neonatal HI.

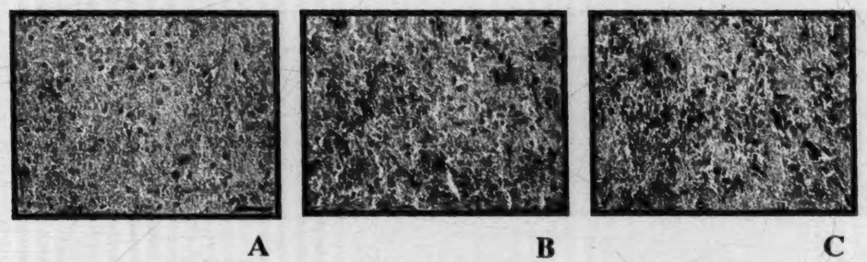

Figure 6.1. ED-1 Immunoreactivity 35 days Post Hypoxia-Ischemia (HI). Representative photomicrographs illustrating that few activated microglia, or ED-1 positive cells exist in the white matter just dorsal to the hippocampus, 35d post-HI. Neither SHAM (A), nor vehicle-treated (B), or doxycyline-treated pups (C) have significantly different numbers of activated microglia. Scale bar = 20μm.

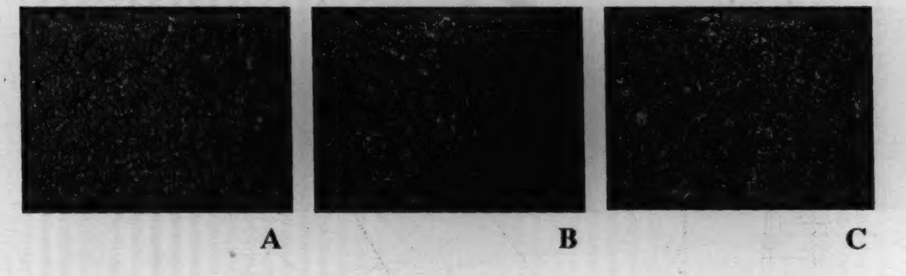

Figure 6.2. GFAP Immunoreactivity 35 days Post Hypoxia-Ischemia (HI). Representative photomicrographs illustrating the typical number of morphology of GFAP-positive astrocytes in the hippocampus 35 days after HI. Numerous reactive astrocytes are present in SHAM (A) and doxycyline-treated pups (C). The astrocytes in vehicle-treated pups are increasingly hypertrophic and have increased expression of GFAP (B). Scale bar = 20μm.

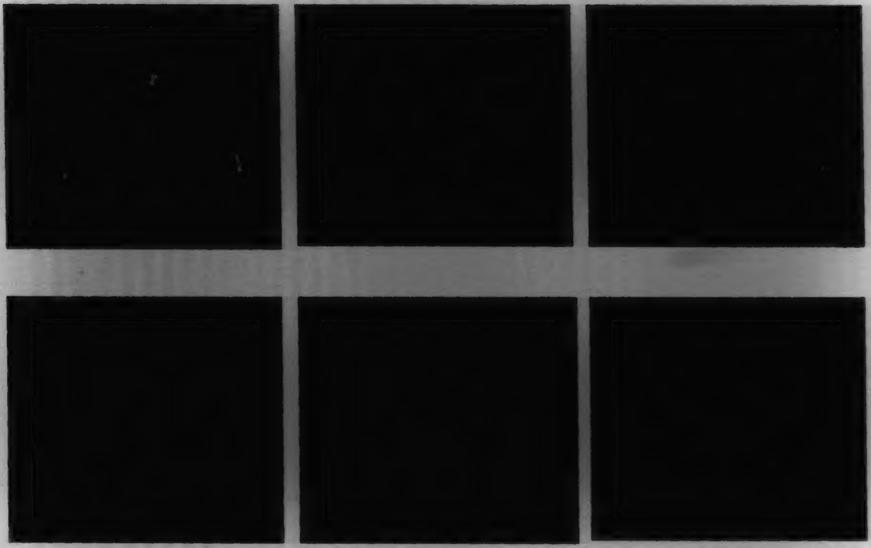

Figure 6.3. Astrocytes are Positive for Interleukin-1β (IL-1β) 35 days Post Hypoxia-Ischemia (HI). Representative photomicrographs showing double immunostaining for (A) GFAP (astrocytes, green); (B) IL-1β (red) in the hippocampus of hypoxic-ischemic rat pups 35d post-H. Plate (C) represents the overlay of images (A) and (B), where astrocytes that are positive for IL-1β appear yellow/orange. Further co-localization experiments reveal that the majority of neurons in the molecular layer of the hippocampus are negative for IL-1β (f). (D) NeuN-positive neurons (green); (E) IL-1β cells (red); (F) overlay of images (D) and (E). Scale bar = 20μm.

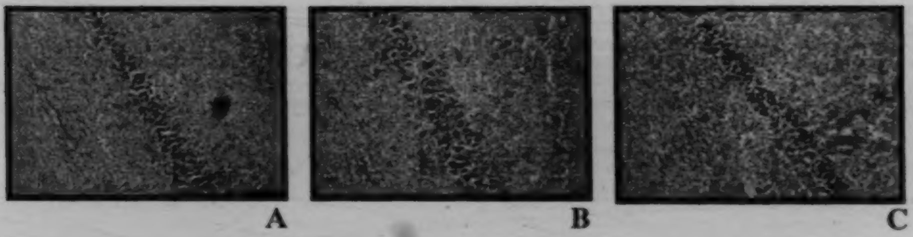

Figure 6.4. Cleaved Caspase-3 Is Still Present 35 days Post Hypoxia-Ischemia (HI). Representative photomicrographs showing immunostaining for cleaved caspase-3 in the hippocampus. Thirty-five days post-HI, significant amounts of cleaved caspase-3 is still present in SHAM (A), vehicle-treated HI (B) and DOXY-treated HI pups (C). Scale bar = 20μm.

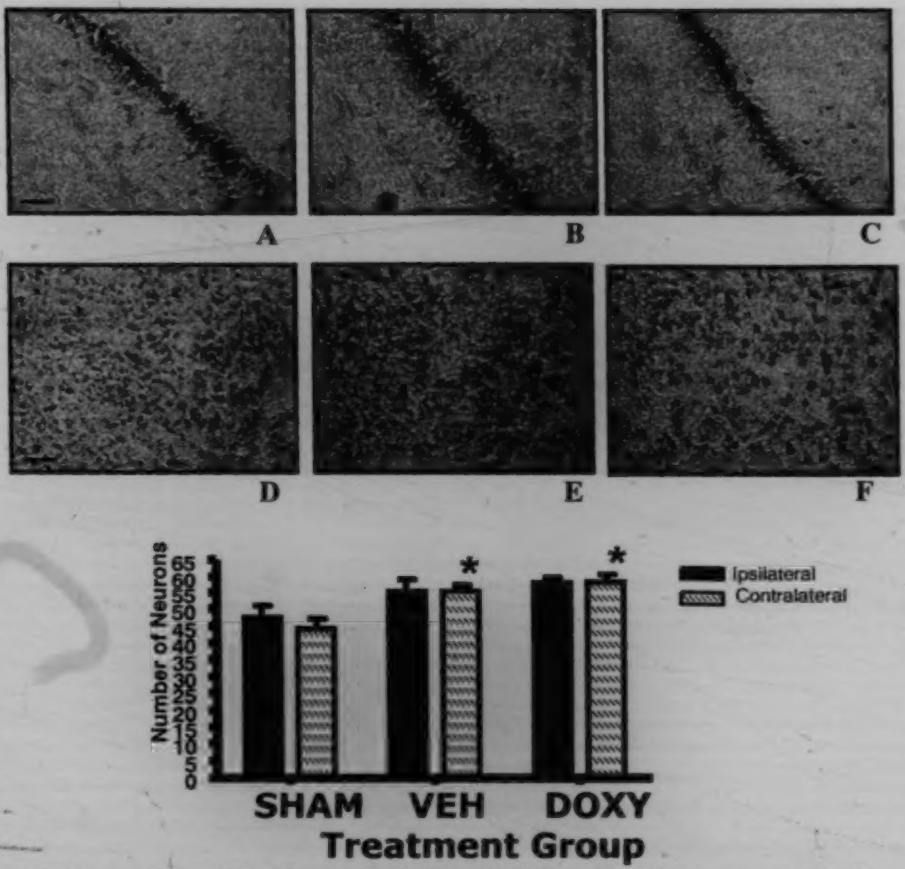
A
B
C
D
E
F
Number of Neurons
65
60
55
50
45
40
35
30
25
20
15
10
5
0
*
*
Ipsilateral
Contralateral
SHAM
VEH
DOXY
Treatment Group

Figure 6.5. Neuronal Cell Survival in the Hippocampus and Cortex 35 days Post Hypoxia-Ischemia (HI). Representative photomicrographs and histograms illustrating neuronal survival 35 days post-HI in the hippocampus (A-C) and dorsal-lateral cortex (D-F). In the hippocampus, vehicle-treated pups appear to have more NeuN-positive neurons (B) than both SHAM (A) and doxycycline-treated HI pups (C). In the dorsal-lateral cortex of VEH-treated pups (E), patchy neuronal cell loss and significant cortical disorganization is evident. DOXY-treated pups (F) have seemingly fewer neurons compared to SHAM (D). Histogram represents the number of neurons counted in medial CA1 both ipsilateral and contralateral to common carotid artery ligation. There are significantly more neurons in medial CA1 of the hippocampus contralateral to common carotid artery liagation in both DOXY and VEH-treated pups compared to SHAM. Scale bars = 20μm.

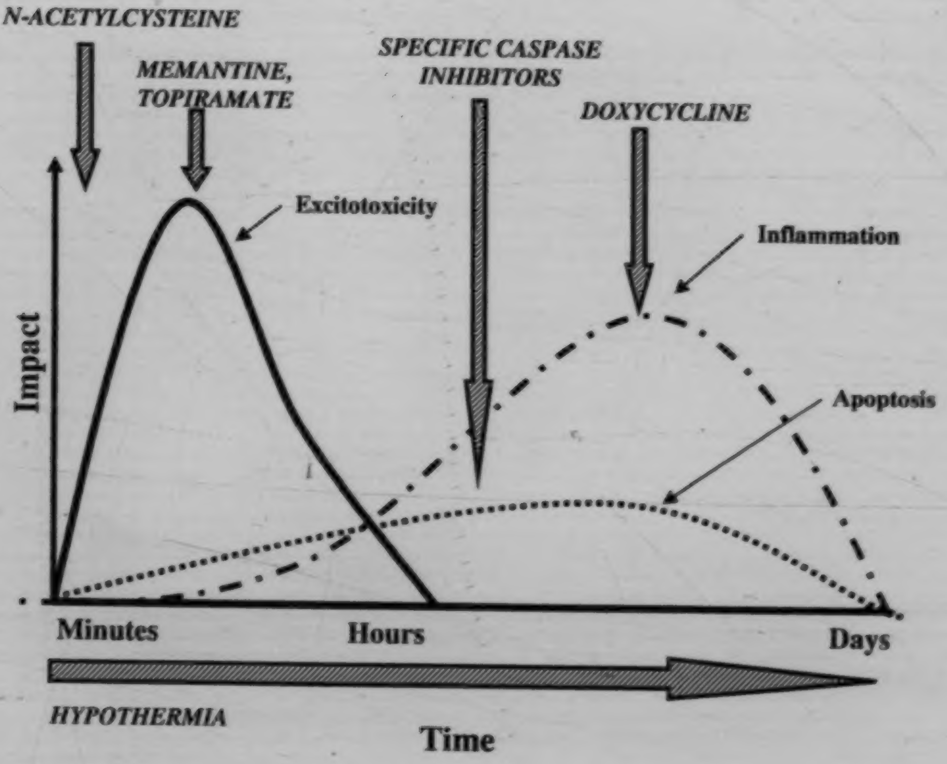
N-ACETYLCYSTEINE
MEMANTINE, TOPIRAMATE
SPECIFIC CASPASE INHIBITORS
DOXYCYCLINE
Excitotoxicity
Inflammation
Apoptosis
Impact
Minutes
Hours
Days
HYPOTHERMIA
Time

Figure 6.6. Putative Treatment Paradigm for Neonatal Hypoxic-Ischemic Brain Injury.

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